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ATTY.'S DOCKET: SLII-P02-001

In re Application of:	)	Art Unit: 1647
POWER et al.	)	Examiner: Regina M. DeBerry
Appln. No.: 10/966,845	)	Washington, D.C.
Date Filed: October 15, 2004	)	Confirmation No. 8075
For: USE OF OSTEOPROTEGERIN…	)	February 27, 2008

DECLARATION UNDER 37 CFR §1.132

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
MS Amendment
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Pierre-Alain VITTE, hereby declare and state as follows:

I am Head of Target Pharmacology at Merck Serono International S.A. in Geneva, Switzerland. My educational and professional experience is presented in the curriculum vitae attached hereto.

The experiments described below evaluate the efficacy of OPG(N)-Fc, which is a fusion protein composed of the N-terminal residues (1-194) of osteoprotegerin (OPG) and the Fc

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portion of human IgG_1 with preserved effector function, in a mouse model of lung fibrosis (as induced by bleomycin) were conducted by me or under my direct supervision and I can attest of my own personal knowledge that all the results of the experiments with OPG(N)-Fc reported herein are true and accurate. The fusion of the Fc portion to the N-terminal part of OPG is used to prolong the half-life of a biological molecule, as is well known in the art.

METHOD

The activity of OPG(N)-Fc was tested according to Example 9 of the application. Lung fibrosis was induced in ketamine/rumpum anesthetized female mice (C57BL/6, 22-25g, Elevage Janvier) by bleomycin sulfate (5 IU/ml) intratracheally administered (20 µl). Body weight was determined daily. Fourteen days after the induction of the disease, the animals were sacrificed. The lungs were weighed before and after drying (overnight in a Speedvac apparatus; see Figs. 1A and 1B). The level of edema in the lungs was estimated by measuring the water content. Fibrosis was assessed by the content of collagen in the lungs, as estimated by the content of hydroxylproline. Hydroxyproline content was determined using the method published by the Association Française de Normalisation - www.afnor.org -

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(NF V 04-415). Briefly, hydroxyproline was released from collagen by acidic hydrolysis (6N HCl, overnight at 110°C), oxidized by chloramine T and coupled with p-dimethylaminobenzaldehyde. The concentration of this compound was determined by spectrophotometry at 558 nm.

OPG(N)-Fc was diluted in phosphate saline buffer (PBS) and administered subcutaneously at a dose of 0.31, 1.25 and 5 mg/kg (once daily, 5 days a week). The first administration was done 4 hours after challenge with bleomycin. Control animals (Ctrl) received PBS (10 ml/kg, sc, once daily, 5 days a week). Sham animals received saline (20 µl intratracheally) and PBS (10 ml/kg, sc, once daily, 5 days a week).

RESULTS

OPG(N)-Fc, administered at a dose of 0.31, 1.25 and 5 mg/kg, significantly reduced bleomycin-induced lung edema (estimated by the water content; see attached Fig. 4) by 15%, 26% and 38%, respectively.

A statistically significant reduction of lung fibrosis (-13%, -33% and -35%, $p<0.05$ and 0.01; see attached Fig. 3) was also observed when OPG(N)-Fc was administered at dose of 0.31, 1.25 and 5 mg/kg, respectively.

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Bleomycin-induced-body weight loss was improved when OPG(N)-Fc was administered at a dose of 0.31, 1.25 and 5 mg/kg (see attached Figs. 1 and 2).

DISCUSSION

OPG(N)-Fc, administered at a dose of 0.31, 1.25 and 5 mg/kg in a preventive/curative dose regimen, significantly reduced edema and fibrosis of the lungs that were induced by bleomycin, and furthermore reduced body weight loss.

This model of bleomycin-induced fibrosis mimics the pathology observed after treatment of cancer with bleomycin. Bleomycin is a compound derived from a fungus that is used as an anticancer agent. A severe side effect of bleomycin treatment is fibrosis. Bleomycin is used in art-recognized animal models to induce fibrosis (Yamamoto T, "The bleomycin-induced scleroderma model: what have we learned for sclerodema pathogenesis?" *Arch Dermatol Res.* 2006 Feb; 297(8):333-441), a copy of which is attached hereto. When applied to the lungs, bleomycin induces lung fibrosis (Hattori et al., "Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice, *J Clin Invest.* 2000 Dec; 106(11):1341-1350), a copy of which is attached hereto. The pathophysiological mechanisms (leukocyte

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infiltration, collagen deposit, etc.) are very similar to those described in primary lung fibrosis.

The data show that the N-terminal region of OPG with the amino acids 22-194 is sufficient to induce the anti-fibrotic effect of OPG (amino acids 1-21 make up the signal peptide, which is cleaved upon secretion of OPG and is therefore not present in the mature OPG protein *in vivo*). Based upon this data and the data in the application demonstrating the anti-fibrotic effect of full-length OPG, I would expect that a peptide comprising amino acids 22-194 or a peptide comprising a sequence having at least 90% identity with amino acids 22-194 also has an anti-fibrotic effect.

The undersigned declares further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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29/02/2008
Date

Pierre-Alain VITTE

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Annex

Fig. 1

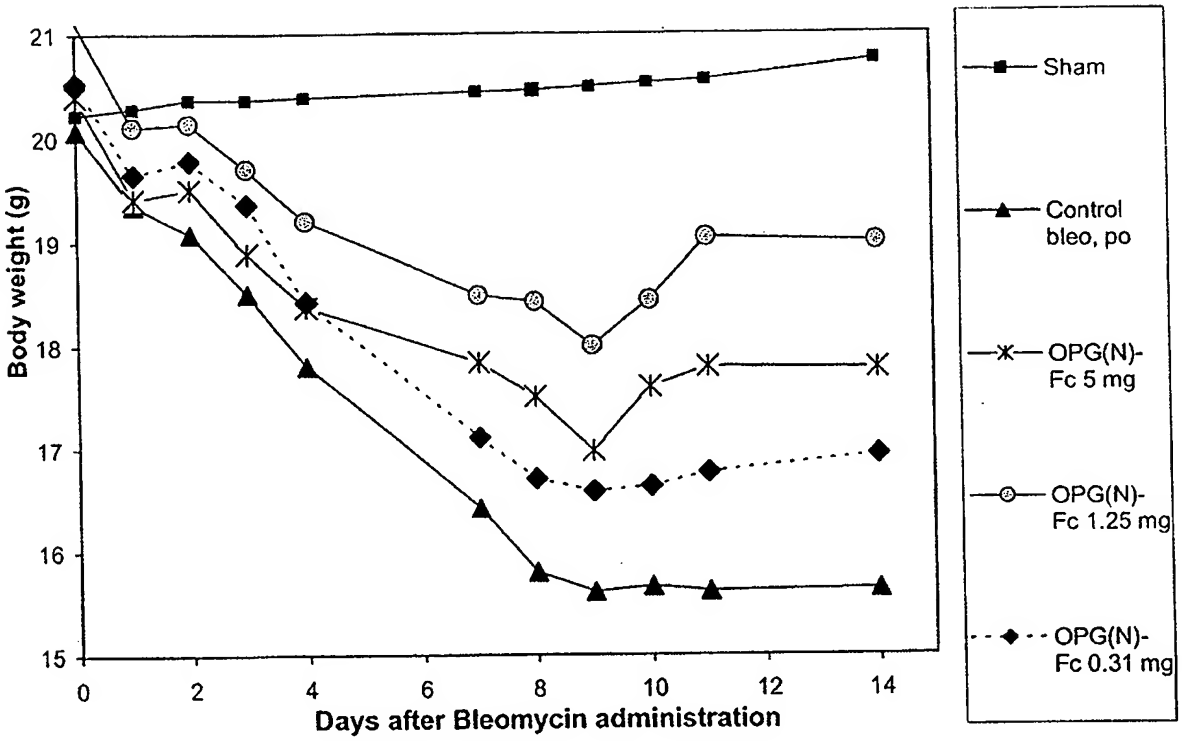

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Fig. 2

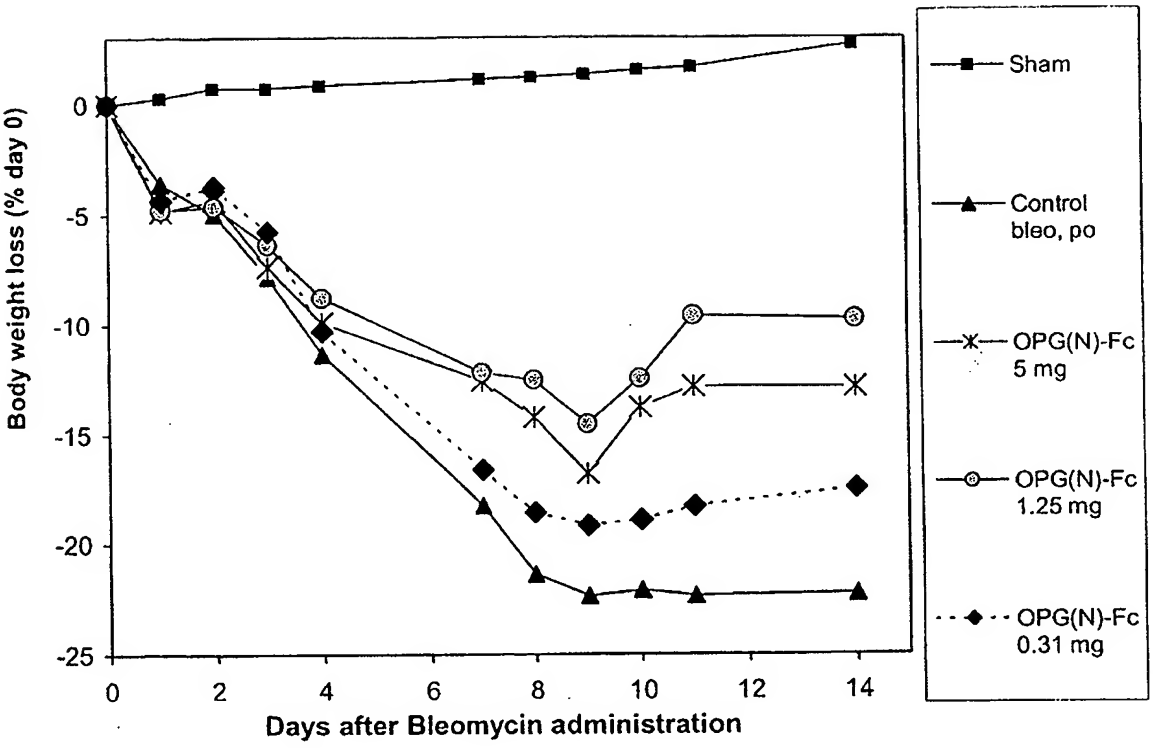

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Fig. 3

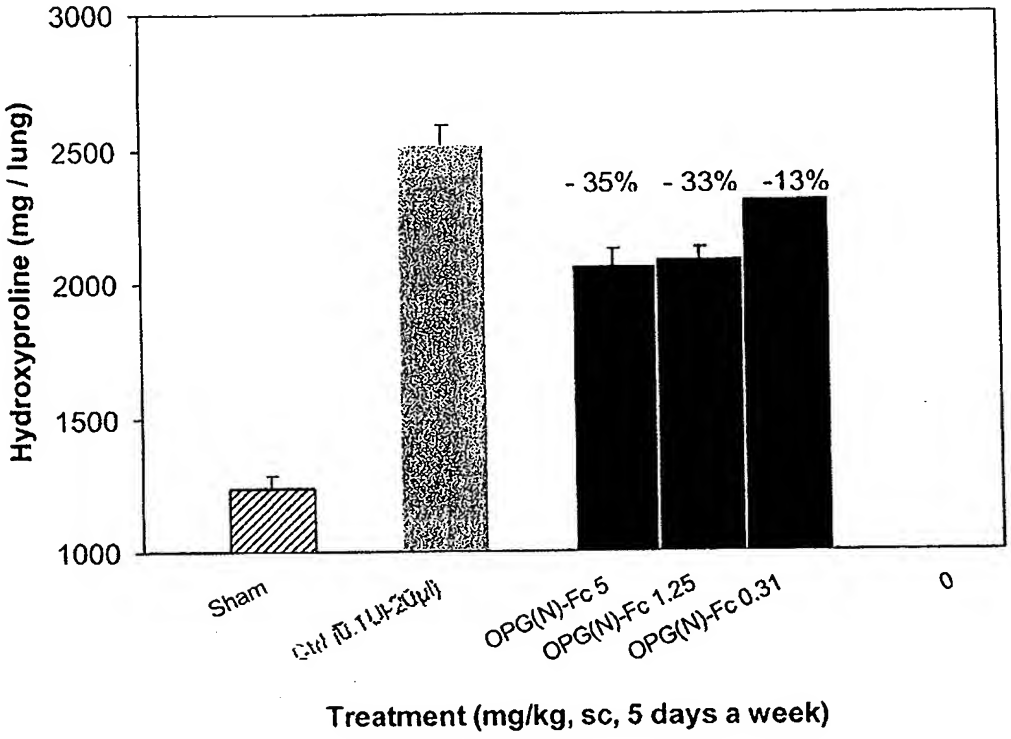

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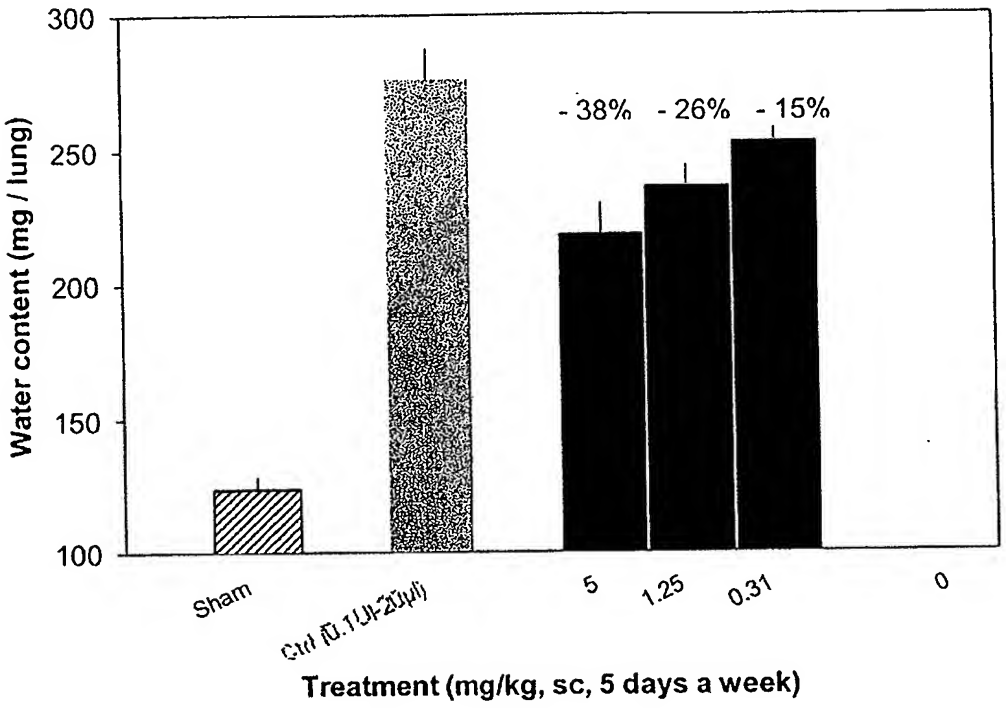
Fig. 4



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CURRICULUM VITAE

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EDUCATION

- Pharmacist, U.F.R. Pharmacie, Lyon, France, 1980
- "Docteur d'Etat ès Sciences Pharmaceutiques" (PhD), U.F.R. Pharmacie, Lyon, 1987
- "Chargé de Recherche INSERM", 1983-85
- "Ancien Interne en Pharmacie des Hôpitaux de Lyon", promotion 1981
- "Diplôme d'Etudes Approfondies de Physiologie", U.F.R. Pharmacie, Lyon, 1981.

PROFESSIONNAL EXPERIENCE

❖ **MERCK SERONO INTERNATIONAL S.A., Geneva (1999-)**

➢ ***Position***: Head of Target Pharmacology

➢ ***Experience and responsibilities***

* Drug discovery

- Therapeutic areas: autoimmune diseases (rheumatoid arthritis, multiple sclerosis, ulcerative colitis), diabetes, neurodegeneration (stroke, neuropathy), chemotaxis / inflammation, oncology (leukemia), preterm labor, safety pharmacology, pharmacokinetics.
- Function
  - Elaboration of the strategy of *in vivo* screening
  - Set-up and validation of animal models
  - Day to day screening of small molecules and proteins
  - Target validation
  - Pharmacokinetics in rodents

* Pharmaceutical development: pharmacologist expert in "Project Teams" (autoimmune diseases, allergy ,diabetes, neurodegeneration, preterm labor).

* Outsourcing of studies (CRO and Universities).

* Management of pharmacokinetics and toxicology studies between Serono centers.

* Animal experimentation: relationships with the Swiss Veterinarian Authorities, member of Serono Ethics Committee for Animal Experimentation.

* Management of an animalry: breeding of transgenic animals in SPF zone; prion zone.

* Basic research: Student programs (Postdoc, PhD, Master).

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❖ **BRACCO RESEARCH SA, Geneva (1993-1999)**

➢ ***Position***: Head of Safety Pharmacology Group

➢ ***Experience and responsibilities***

* Safety Pharmacology: cardiovascular, respiration, CNS, immunology, hematology and coagulation (GLP studies).

* Basic Research: study of the physiological and biochemical mechanisms of the side effects of particles administered by parenteral routes.

* Pharmaceutical Development: expert in safety pharmacology in the pre-development programs of contrast media for echography and X ray Scan.

❖ **BATTELLE MEMORIAL INSTITUTE, Geneva (1988-1993)**

➢ ***Position***: Research Scientist in Contract Research Service.

➢ ***Experience and responsibilities***

* Study Director

- Brain imaging, cerebral ischemia, behavior pharmacology (memory and depression).
- Safety pharmacology, pharmacokinetics, endocrinology.
- Receptor binding (*in vitro* and *in vivo*).

* Marketing / Sales

❖ **INSERM U171, Groupe de Neurochimie Fonctionnelle, Lyon (1983-1985)**

➢ ***Position***: "Chargé de recherche" INSERM.

➢ ***Experience***

* Functional Neuroanatomy and cerebral metabolism

* Neuroendocrinology.

* PET Scan

❖ **INTERNAT EN PHARMACIE, Lyon (1981-1988)**

➢ ***Position***: Interne en Pharmacie.

➢ ***Location***: Pharmaceutical Services of "Hospices Civils de Lyon".

➢ ***Experience***: Clinical toxicology, clinical biochemistry, pharmacokinetics, nuclear medicine, analytical chemistry (chromatography, radioimmunology), microbiology.

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5-HT1A, 5-HT1B, 5-HT1C, 5-HT1D et 5-HT3, pour Laboratoires Anphar Rolland, M. Guerrier, T. Filloux et P.A. Vitte, Août 1989.

19. ETUDE NEUROCHIMIQUE ET NEUROPHARMACOLOGIQUE DE LA MEDIFOXAMINE, Partie II: ETUDE, IN VIVO, DE LA LIAISON DE LA MEDIFOXAMINE AUX RECEPTEURS 5-HT2, pour Laboratoires Anphar Rolland, M. Guerrier, T. Filloux et P.A. Vitte, Août 1989.
20. ETUDE NEUROCHIMIQUE ET NEUROPHARMACOLOGIQUE DE LA MEDIFOXAMINE, Partie III: ETUDE, IN VITRO, DE LA LIAISON DE LA MEDIFOXAMINE AUX RECEPTEURS 5-HT2 PAR RADIOAUTOGRAPHIE QUANTITATIVE, pour Laboratoires Anphar Rolland, M. Guerrier, T. Filloux et P.A. Vitte, Août 1989.
21. ETUDE NEUROCHIMIQUE ET NEUROPHARMACOLOGIQUE DE LA MEDIFOXAMINE, Partie IV : ETUDE, EX VIVO, DES EFFETS D'UN TRAITEMENT SUB-CHRONIQUE DE LA MEDIFOXAMINE SUR LE RECAPTAGE DE LA 3H-5-HT DANS LE CERVEAU DE RAT, pour Laboratoires Anphar Rolland, M. Guerrier, T. Filloux et P.A. Vitte, Août 1989.
22. ETUDE NEUROCHIMIQUE ET NEUROPHARMACOLOGIQUE DE LA MEDIFOXAMINE: ETUDE, IN VIVO, DES EFFETS DE LA MEDIFOXAMINE SUR LA LIBERATION DE LA 5-HT, DE LA DOPAMINE ET DE LEURS METABOLITES PAR MICRODIALYSE CHEZ LE RAT LIBRE DE SE MOUVOIR, pour Laboratoires Anphar Rolland, T. Filloux, M. Guerrier, P.F. Curle et P.A. Vitte, Août 1989.
23. LIAISON DU 3H-SPIPERONE EX VIVO: ETUDE DE L'ACCUMULATION DU 3H-SPIPERONE INDUITE PAR LE PIRIBEDIL, Partie I: REGULATION DU RECEPTEUR D2, pour I.R.I.S., M. Guerrier et P.A. Vitte, Novembre 1989.
24. LIAISON DU 3H-SPIPERONE EX VIVO: ETUDE DE L'ACCUMULATION DU 3H-SPIPERONE INDUITE PAR LE PIRIBEDIL, Partie II: DEBIT SANGUIN CEREBRAL LOCAL, pour I.R.I.S., M. Guerrier et P.A. Vitte, Novembre 1989.
25. LIAISON DU 3H-SPIPERONE EX VIVO: ETUDE DE L'ACCUMULATION DU 3H-SPIPERONE INDUITE PAR LE PIRIBEDIL, Partie III: ETUDE, EX VIVO, DE LA FIXATION DU 3H-SPIPERONE PAR RADIOAUTOGRAPHIE QUANTITATIVE, pour I.R.I.S., M. Guerrier et P.A. Vitte, Novembre 1989.
26. ETUDE DES EFFETS DU LM 94 SUR LES RECEPTEURS 5-HT UPTAKE ET VIP, pour LIPHA, T. Filloux et P.A. Vitte, Novembre 1989.
27. THE EFFECTS OF ZB - 1 ON BRAIN ISCHEMIA IN GERBILS, for Zeria Pharm.Co. Ltd., M. Guerrier et P.A. Vitte, November 1989.
28. INTERACTION OF EXTRACTS OF VALERIANA AND HYPERICUM WITH THE GABAA / BENZODIAZEPINE RECEPTOR / Cl- IONOPHORE IN MICE IN VIVO, Part I: EFFECT OF VALERIANA AND HYPERICUM ON BENZODIAZEPINE RECEPTOR BINDING IN VIVO, for Steiner Arzneimittel, D. Larue, M. Guerrier, T. Filloux and P.A. Vitte, November 1989.
29. INTERACTION OF EXTRACTS OF VALERIANA AND HYPERICUM WITH THE GABAA / BENZODIAZEPINE RECEPTOR / Cl- IONOPHORE IN MICE IN VIVO, Part II: EFFECT OF VALERIANA AND HYPERICUM ON THE STEADY STATE LEVELS OF 3H-GABA BRAIN/PLASMA RATIO AFTER I.P. ADMINISTRATION OF 3H-GABA TO MICE, for Steiner Arzneimittel, D. Larue, M. Guerrier, T. Filloux and P.A. Vitte, November 1989.
30. ETUDE, IN VITRO, DES EFFETS DE LA MEDIFOXAMINE SUR LA PHOSPHOLIPASE C, pour Laboratoires Anphar-Rolland, D. Larue, M. Guerrier, T. Laforge et P.A. Vitte, Décembre 1989.
31. INTERLEUKIN-2 AND GM-CSF INDIRECT ELISA, for Glaxo IMB, T. Filloux, M. Guerrier, and P.A. Vitte, December 1989.
32. THE EFFECTS OF JO 1784 ON BRAIN ISCHEMIA IN GERBILS, for Jouveinal Laboratoires, M. Guerrier et P.A. Vitte, December 1989.
33. THE EFFECTS OF F-721 ON BRAIN ISCHEMIA IN GERBILS, Phase I, for Norwich Eaton Pharmaceuticals INC., T. Filloux and P.A. Vitte, January 1990.

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34. THE EFFECTS OF F-721 ON BRAIN ISCHEMIA IN GERBILS, Phase 2, for Norwich Eaton Pharmaceuticals, INC., T. Filloux and P.A. Vitte, March 1990.

35. ETUDE DES EFFETS NOOTROPES DU LDS-008 ET DU LDS-009, pour SERPA M. Garcia, P.F. Curle et P.A. Vitte, Mars 1990.

36. PENETRATION OF 3H-VINPOCETINE THROUGH THE BLOOD BRAIN BARRIER AND AUTORADIOGRAPHIC LOCALIZATION, for Thiemann Arzneimittel GmbH, M. Guerrier and P.A. Vitte, March 1990.

37. RECEPTOR AND SECOND MESSENGER SYSTEMS IN PERIPHERAL ORGANS IN EXPERIMENTAL DIABETIC NEUROPATHY: EFFECT OD DTR 100, for Depha Team, T. Filloux and P.A. Vitte, March, 1990.

38. ETUDE RADIOAUTOGRAPHIQUE DE LA DISTRIBUTION TISSULAIRE DU [14C]-R.7621, pour Laboratoires Debat, D. Larue et P.A. Vitte, Avril 1990.

39. SCREENING ETUDE DE 3 GROUPES DE PRODUITS pour I.R.I.S., N. Giessinger, T. Filloux, A. Broillet, P.F. Curle, X. Fouillet et P.A. Vitte, Juin 1990.

40. EFFECTS OF EXTRACTS OF VALERIANA AND HYPERICUM, PART I: ON GABA-TRANSAMINASE ACTIVITY IN VITRO, for Steiner Arzneimittel, D. Larue and P.A. Vitte, June 1990.

41. EFFECTS OF EXTRACTS OF VALERIANA AND HYPERICUM, PART II: ON BENZODIAZEPINE RECEPTOR BINDING ACTIVITY IN VITRO, for Steiner Arzneimittel, D. Larue and P.A. Vitte, June 1990.RECEPTOGRAM SCREEN S 9977-2 AND S 14287-1, for I.R.I.S., T. Filloux and P.A. Vitte, August 1990.

42. NEUROCHEMICAL STUDIES ON ACETYL-L-CARNITINE, Part I: DETERMINATION OF AGONIST/ANTAGONIST/INVERSE AGONIST PROPERTIES IN VITRO, for Sigma - Tau Industrie Farmaceutiche Riunite, V. Fraimbault and P.A. Vitte, August, 1990.

43. NEUROCHEMICAL STUDIES ON ACETYL-L-CARNITINE, Part II: Cl- FLUX EXPERIMENTS, for Sigma - Tau Industrie Farmaceutiche Riunite, V. Fraimbault and P.A. Vitte, August, 1990.

44. STUDIES OF THE EFFECTS OF S 9977-2 AND S 14287-1 ON ACETYLCHOLINESTERASE ACTIVITY IN RAT BRAIN, for I.R.I.S., D. Larue, M. Guerrier et P.A. Vitte, August 1990.

45. EFFECTS OF FIVE COMPOUNDS IN FOUR RECEPTOR BINDING ASSAYS, for Jouveinal Laboratoires, T. Filloux and P.A. Vitte, September 1990.

46. DEVELOPPEMENT D'UN DOSAGE RADIOIMMUNOLOGIQUE DE L'ACETYLHOMOTAURINE, PRODUCTION D'UN CONJUGUE IMMUNOGENE, pour LIPHA, M. Guerrier and P.A. Vitte, Septembre 1990.

47. EFFETS DU RU 23 908 SUR LA CONSOMMATION ALIMENTAIRE DU RAT MALE, pour Roussel-Uclaf, C. Ventrone, T. Filloux et P.A. Vitte, Novembre 1990.

48. BRAIN ISCHEMIA IN MONGOLIAN GERBILS: THE EFFECTS OF 8 DRUGS (ZBT-5, ZBT-6, ZBT-7, ZBT-8, ZBT-9, ZBT-10, ZBT-11, ZBT-12), for Zeria Pharm.Co. Ltd., V. Fraimbault and P.A. Vitte, November 1990.

49. RECEPTOGRAM SCREEN, for Glaxo Group Research Ltd., P.-A. Vitte, S. Rahali and T. Filloux, November 1990.

50. NEUROPHARMACOLOGICAL STUDIES OF 2 DRUGS, Phase I: BRAIN ISCHEMIA IN MONGOLIAN GERBILS AND PASSIVE AVOIDANCE TEST, for Duphar B. V., T. Filloux and P.A. Vitte, November 1990.

51. NEUROPHARMACOLOGICAL STUDIES OF 2 DRUGS, Phase II: HYPOXIA IN MONGOLIAN GERBILS AND PASSIVE AVOIDANCE TEST, for Duphar B. V., T. Filloux and P.A. Vitte, November 1990.

52. INTERLEUKIN-2 AND GM-CSF ASSAYS IN HUMAN SERA, PHASE I: VALIDATION OF ASSAY, for Glaxo IMB, D. Larue, and P.A. Vitte, December 1990.

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53. INTERLEUKIN-2 AND GM-CSF INDIRECT ELISA, PHASE II: APPLICATION TO CLINICAL TRIALS, for Glaxo IMB, D. Larue, and P.A. Vitte, December 1990.

54. INTERLEUKIN-2 AND GM-CSF ASSAYS IN HUMAN SERA, PHASE II: APPLICATION TO CLINICAL TRIALS, for Glaxo IMB, D. Larue, and P.A. Vitte, December 1990.

55. CYCLANDELATE RECEPTOGRAM SCREEN, for 3M Medica, V. Fraimbault, F.Delean and P.A. Vitte, February 1991.

56. ETUDE BIOCHIMIQUE IN VITRO DU S-9977-2, EFFECT D'UN TRAITEMENT SUBCHRONIQUE PAR LE S-9977-2 SUR LA DENSITE ET L'AFFINITE DES RECEPTEURS MUSCARINIQUES M1 ET M2 ET NICOTINIQUES DANS LE CERVEAU DE RAT, pour I.R.I.S., T. Filloux et P.A. Vitte, Mars 1991.

57. NEUROPHARMACOLOGICAL STUDIES WITH 2 MADAUS COMPOUNDS, Part II : EFFECT OF Fh-27 HCl ON LOCAL CEREBRAL BLOOD FLOW: DETERMINATION USING THE [14C]-IODOANTIPYRINE METHOD, for Madaus A.G., T. Filloux and P.A. Vitte, March 1991.

58. ETUDE BIOCHIMIQUE IN VITRO DU S-9977-2, EFFETS DU S-9977-2 SUR LES SECONDS MESSAGERS COUPLES AUX RECEPTEURS MUSCARINIQUES M1 ET M2, pour I.R.I.S., T. Filloux et P.A. Vitte, Avril 1991.

59. RECEPTOGRAM DU S-12361, pour I.R.I.S., V. Fraimbault , F. Deléan et P.A. Vitte, Avril 1991.

60. STUDY OF THE EFFECTS OF 6 DRUGS OF JOUVEINAL LABORATORIES ON 3 RECEPTORS, for Jouveinal Laboratoires, V. Fraimbault and P.A. Vitte, Avril 1991.

61. STUDY OF THE EFFECTS OF PER OS ADMINISTRATION OF S-20499 ON BLOOD PRESSURE IN AWAKE RATS, for I.R.I.S. et Compagnie - Développement, P.A. Vitte, May 1991.

62. STUDY OF THE EFFECTS OF PER OS ADMINISTRATION OF S-20500 ON BLOOD PRESSURE IN AWAKE RATS, for I.R.I.S. et Compagnie - Développement, P.A. Vitte, May 1991.

63. STUDY OF THE EFFECTS OF S.C. ADMINISTERED S-20500 AND S-20499 ON BLOOD PRESSURE IN AWAKE RATS, for I.R.I.S. et Compagnie - Développement, P.A. Vitte, May 1991.

64. RECEPTOGRAM, pour I.R.I.S. et Compagnie - Développement, F. Deléan, V. Fraimbault et P.A. Vitte, Juin 1991.

65. ETUDE PHARMACOLOGIQUE DU TX-194, Partie I : DETERMINATION DE LA DL50 CHEZ LA SOURIS PAR VOIE ORALE, pour Laboratoires Theramex, C. Delaporte, V. Fraimbault et P.A. Vitte, Juin 1991.

66. ETUDE PHARMACOLOGIQUE DU TX-194, Partie II : EFFETS DE L'ADMINISTRATION PER OS DU TX-194 ET DE LA CETIRIZINE SUR LE BRONCHOSPASME INDUIT PAR L'HISTAMINE CHEZ LE COBAYE, pour Laboratoires Theramex, C. Delaporte, V. Fraimbault et P.A. Vitte, Juin 1991.

67. ETUDE PHARMACOLOGIQUE DU TX-194, Partie III : RECEPTOGRAM, pour Laboratoires Theramex, C. Delaporte, V. Fraimbault et P.A. Vitte, Juin 1991.

68. ETUDE DES EFFETS DE DEUX PRODUITS SUR LA TYROSINE-HYDROXYLASE, LA PHENYLETHANOLAMINE-N-METHYLTRANSFERASE, LA DOPAMINE-§-HYDROXYLASE ET LA CATECHOL-O-METHYLTRANSFERASE DANS LE CERVEAU DE RAT, pour Sodepharm, S. Bouteille et P.A. Vitte, Juin 1991.

69. ETUDE DE 9 PRODUITS ET ADDENDA I-II, pour I.R.I.S. et Compagnie - Développement, C. Delaporte, T. Filloux et P.A. Vitte, Juin 1991.

70. ETUDE DES EFFETS DU PIRIBEDIL DANS UN MODELE DE LA MALADIE D'ALZHEIMER (LESION DU NBM), pour I.R.I.S., A. Prévot et P.A. Vitte, Août 1991.

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71. RECEPTOGRAM™ DU S-14280-1, pour I.R.I.S. et Compagnie - Développement, F. Deléan, S. Bouteille et P.A. Vitte, Août 1991.

72. SCREENING ETUDE DE 9 PRODUITS, ADDENDA III-IV, pour I.R.I.S. et Compagnie - Développement, S. Bouteille, T. Filloux et P.A. Vitte, Août 1991.

73. ETUDE DES EFFETS DE DIFFERENTES DROGUES SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, pour I.R.I.S. et Compagnie - Développement, A. Prévot et P.A. Vitte, Août 1991.

74. RECEPTOGRAM, DETERMINATION DES IC50 PRECISES (Série M. Devissaguet), pour I.R.I.S. et Compagnie - Développement, S. Bouteille et P.A. Vitte, Septembre 1991.

75. RECEPTOGRAM, DETERMINATION DES IC50 PRECISES (Série B. Guardiola),pour I.R.I.S. et Compagnie - Développement, S. Bouteille et P.A. Vitte, Septembre 1991.

76. EFFECTS OF ZB-P-1 ON PROTEIN SYNTHESIS IN VITRO, for Zeria, S. Bouteille and P.A. Vitte, November 1991.

77. ETUDE DES EFFETS DE SEPT PRODUITS SUR L'ACTIVITE LOCOMOTRICE ET LES CAPACITES MNESIQUES DE LA GERBILLE, Partie I: ETUDE DES EFFETS DE SEPT PRODUITS SUR L'ACTIVITE LOCOMOTRICE DE LA GERBILLE, pour I.R.I.S. et Compagnie - Développement, C. Delaporte, T. Filloux et P.A. Vitte, Novembre 1991.

78. ETUDE DES EFFETS DE SEPT PRODUITS SUR L'ACTIVITE LOCOMOTRICE ET LES CAPACITES MNESIQUES DE LA GERBILLE, Partie II: ETUDE CHEZ LA GERBILLE DES EFFETS DE SEPT PRODUITS SUR L'AMNESIE INDUITE PAR SCOPOLAMINE, pour I.R.I.S. et Compagnie - Développement, C. Delaporte, T. Filloux et P.A. Vitte, Novembre 1991.

79. RECEPTOGRAM™, DETERMINATION DES IC50 DE LA LIAISON DU S-14280-1 AUX RECEPTEURS ADRENERGIQUES alA ET alB ET 5-HT2, pour I.R.I.S. et Compagnie - Développement, S. Bouteille et P.A. Vitte, Novembre 1991.

80. ETUDE DES EFFETS DE DIFFERENTES DROGUES SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, ADDENDA III-IV, pour I.R.I.S. et Compagnie - Développement, A. Prévot et P.A. Vitte, Septembre 1991.

81. ETUDE DE 9 PRODUITS, ADDENDA V-VI, pour I.R.I.S. et Compagnie - Développement, S. Bouteille, T. Filloux et P.A. Vitte, Novembre 1991.SCREENING ETUDE DE 9 PRODUITS, ADDENDA VII-VIII, pour I.R.I.S. et Compagnie - Développement, S. Bouteille, T. Filloux et P.A. Vitte, Novembre 1991.

82. EFFECTS OF ZB-P-1 ON PROTEIN SYNTHESIS IN VITRO, for Zeria, S. Bouteille and P.A. Vitte, January 1992.

83. NEUROPHARMACOLOGICAL STUDIES OF DUPHAR COMPOUND D, FOCAL ISCHEMIA BY MCA OCCLUSION IN MICE AND HISTOLOGY, for Duphar B. V., V. Fraimbault and P.A. Vitte, January 1992.

84. NEUROPHARMACOLOGICAL STUDIES OF DUPHAR COMPOUND S, Part I: HYPOXIA IN MONGOLIAN GERBILS AND PASSIVE AVOIDANCE TEST, or Duphar B. V., V. Fraimbault and P.A. Vitte, January 1992.

85. NEUROPHARMACOLOGICAL STUDIES OF DUPHAR COMPOUND S, Part II: FOCAL ISCHEMIA BY MCA OCCLUSION IN MICE AND HISTOLOGY, for Duphar B. V., V. Fraimbault and P.A. Vitte, January 1992.

86. ETUDE EX VIVO DE LA LIAISON DU PRAZEPAM, DU BROMAZEPAM ET DE L'ALPRAZOLAM AUX RECEPTEURS DES BENZODIAZEPINES, pour Parke - Davis, S. Bouteille et P.A. Vitte, Janvier 1992.

87. ETUDE DES EFFETS DE SEIZE PRODUITS UCB SUR LES RECEPTEURS HISTAMINE H1 PULMONAIRES, pour UCB, A. Prévot et P.A. Vitte, Janvier 1992.

88. AMNESIE A LA SCOPOLAMINE ET EVITEMENT PASSIF, ETUDE DE DEUX PRODUITS (RPT 109 et RPT 110) DES LABORATOIRES DELAGRANGE, pour Laboratoires Delagrange, A. Prévot et P.A. Vitte, Janvier 1992.

89. RECEPTOGRAM™, pour Laboratoires Delagrange, C. Delaporte, V. Fraimbault and P.A. Vitte, Janvier 1992.

90. STUDY OF THE EFFECTS OF I.P. ADMINISTERED S-20098 ON BLOOD PRESSURE IN PENTOBARBITAL ANESTHETIZED RATS, for I.R.I.S. et Compagnie - Développement, T. Filloux and P.A. Vitte, January 1992.

91. ETUDE DES EFFETS DU PIRIBEDIL DANS UN MODELE DE LA MALADIE D'ALZHEIMER (AF64A), pour IRIS, A. Prévot et P.A. Vitte, Janvier 1992.

92. ETUDE DES EFFETS DE DIFFERENTES DROGUES SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, ADDENDA V-VI, pour I.R.I.S. et Compagnie - Développement, A. Prévot et P.A. Vitte, Janvier 1992.

93. ETUDE DES EFFETS DE DIFFERENTES DROGUES SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, ADDENDA VII-VIII, pour I.R.I.S. et Compagnie - Développement, A. Prévot et P.A. Vitte, Janvier 1992.

94. STUDY OF THE TISSUE DISTRIBUTION OF [14C-4]-CLOPIDOGREL, QUALITATIVE STUDY, for Sanofi Recherche, by S. Bouteille and P.A. Vitte, February 1992.

95. STUDY OF THE TISSUE DISTRIBUTION OF [14C-4]-CLOPIDOGREL, QUANTITATIVE STUDY, for Sanofi Recherche, S. Bouteille and P.A. Vitte, February 1992.

96. GABA ANALYSIS BY HPLC OF VALERIANA, VCEF 6 & VCEF 6/1, for Steiner Arzneimittel, P.A. Vitte, February 1992.

97. ETUDE DES EFFETS D'UNE DROGUE SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, ADDENDA IX, pour I.R.I.S. et Compagnie - Développement, S. Bouteille et P.A. Vitte, Mars 1992.

98. ETUDE DES EFFETS D'UNE DROGUE SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, ADDENDA X, pour I.R.I.S. et Compagnie - Développement, A. Prévot et P.A. Vitte, Mars 1992.

99. RECEPTOGRAM™ (4 produits), pour I.R.I.S., S. Bouteille, F. Delaporte, V. Fraimbault et P.A. Vitte, Mars 1992.

100. HYPOXIE NORMOBARE CHEZ LA SOURIS, ETUDE D'UN PRODUIT JOUVEINAL, pour Institut de Recherche Jouveinal, A. Prévot et P.A. Vitte, Mars 1992.

101. HYPOXIE NORMOBARE CHEZ LA SOURIS, ETUDE D'UN PRODUIT JOUVEINAL, ADDENDA I, Institut de Recherche Jouveinal, A. Prévot et P.A. Vitte, Avril 1992.

102. MUSCARINIC EFFECTS OF S-9977-2, Part I: INTERACTION OF S-9977-2 WITH MUSCARINIC RECEPTORS IN VIVO, for I.R.I.S., A. Prévot and P.A. Vitte, April 1992.

103. MUSCARINIC EFFECTS OF S-9977-2, Part II: IN VITRO AND IN VIVO EFFECTS OF S-9977-2 ON THE BINDING OF [3H]-QNB TO SERUM PROTEINS for I.R.I.S., A. Prévot and P.A. Vitte, April 1992.

104. MUSCARINIC EFFECTS OF S-9977-2, Part III: EFFETS OF AN ACUTE TREATMENT OF S-9977-2 ON MUSCARINIC RECEPTORS IN BRAIN RAT, for I.R.I.S., A. Prévot and P.A. Vitte, Avril 1992.

105. ETUDE DES EFFETS D'UNE DROGUE SUR UN MODELE D'HYPOXIE NORMOBARE CHEZ LA SOURIS, ADDENDA XI, pour I.R.I.S. et Compagnie - Développement, A. Prévot et P.A. Vitte, Avril 1992.

106. HYPOXIE NORMOBARE CHEZ LA SOURIS, Addenda XII : Etude de 5 produits, pour I.R.I.S. et Compagnie - Développement, V. Fraimbault et P.A. Vitte, Juin 1992.

107. HYPOXIE NORMOBARE CHEZ LA SOURIS, Addenda XIII : Etude de 6 produits, pour I.R.I.S. et Compagnie - Développement, V. Fraimbault et P.A. Vitte, Juin 1992.

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108. ETUDE DE LA LIAISON DU TX194 ET DE LA TERFENADINE A 4 RECEPTEURS, pour Théramex, B. Françon et P.A. Vitte, Juin 1992.
109. ETUDE DES PROPRIETES CHOLINERGIQUES DU PIRIBEDIL, pour I.R.I.S., T. Filloux et P.A. Vitte, Octobre 1992.
110. RECEPTOGRAM™ S-20499 (Volumes 1 & 2), for I.R.I.S., V. Fraimbault, B. Françon and P.A. Vitte, September 1992.
111. RECEPTOGRAM™ S-20098 (Volumes 1 & 2), for I.R.I.S., V. Fraimbault, B. Françon and P.A. Vitte, September 1992.
112. RECEPTOGRAM™ S-14506 (Volumes 1 & 2), for I.R.I.S., V. Fraimbault, B. Françon and P.A. Vitte, September 1992.
113. RECEPTOGRAM™ BRACCO 1992, for Bracco S.p.A., F. Deléan, T. Filloux and P.A. Vitte, September 1992.
114. RECEPTOR BINDING PROFILE OF CHELIDONIUM AND EXTRACTS, for Steiner Arzneimittel, S. Bardonnet, V. Fraimbault and P.A. Vitte, September 1992.
115. ETUDE DES EFFETS NEUROPROTECTEURS DU SR 57746A, Partie I : Hypoxie normobare chez la souris, pour Sanofi Recherche, T. Filloux and P.A. Vitte, Décembre 1992.
116. ETUDE DES EFFETS NEUROPROTECTEURS DU SR 57746A, Partie II : Hypoxie normobare et évitement passif chez la gerbille, pour Sanofi Recherche, T. Filloux and P.A. Vitte, Décembre 1992.
117. ETUDE DES EFFETS NEUROPROTECTEURS DU SR 57746A, Partie III : Ischémie globale chez la gerbille, pour Sanofi Recherche, T. Filloux and P.A. Vitte, Décembre 1992.
118. DETERMINATION OF IC50 VALUES OF A SERIES OF LTM COMPOUNDS ON BRAIN HISTAMINE H1 RECEPTORS & HUMAN AND RABBIT PLATELET PAF RECEPTORS, for Centre de Recherche L.T.M., F. Deléan and P.A. Vitte, January 1993.
119. STUDY OF S-21111, S-21157 & I-5464 ON BLOOD PRESSURE IN AWAKE RATS, for I.R.I.S., T. Filloux and P.A. Vitte, February 1993.
120. STUDY OF THE EFFECTS OF VALERIANA AND HYPERICUM ON THE GABA-BENZODIAZEPINE RECEPTOR COMPLEX, Part I: Effects of Valeriana , Hypericum and Valeriana + Hypericum on the GABA-Benzodiazepine receptor complex in vitro, for Steiner Arzneimittel, F. Deléan and P.A. Vitte, February 1993.
121. STUDY OF THE EFFECTS OF VALERIANA AND HYPERICUM ON THE GABA-BENZODIAZEPINE RECEPTOR COMPLEX, Part II: Effects of Hypericum and a Valeriana + Hypericum mixture on Benzodiazepine receptors in vivo, for Steiner Arzneimittel, F. Deléan and P.A. Vitte, February 1993.
122. STUDY OF THE EFFECTS OF VALERIANA AND HYPERICUM ON THE GABA-BENZODIAZEPINE RECEPTOR COMPLEX, Part III: Effects of Valeriana and Hypericum on GABA uptake in rat brain synaptosomes, for Steiner Arzneimittel, F. Deléan and P.A. Vitte, February 1993.
123. BINDING OF NAE-086 TO 5-HT1B 5-HT1C & 5-HT3 RECEPTORS, for Astra Arcus AB, F. Deléan and P.A. Vitte, March 1993.
124. ISCHEMIE FOCALE CHEZ LA SOURIS, Etude du S-20595, pour I.R.I.S., V. Fraimbault et P.A. Vitte, Mars 1993.
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Bleomycin-induced pulmonary fibrosis in fibrinogen-null mice

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Mice deleted for the plasminogen activator inhibitor-1 (*PAI-1*) gene are relatively protected from developing pulmonary fibrosis induced by bleomycin. We hypothesized that PAI-1 deficiency reduces fibrosis by promoting plasminogen activation and accelerating the clearance of fibrin matrices that accumulate within the damaged lung. In support of this hypothesis, we found that the lungs of *PAI-1*$^{-/-}$ mice accumulated less fibrin after injury than wild-type mice, due in part to enhanced fibrinolytic activity. To further substantiate the importance of fibrin removal as the mechanism by which PAI-1 deficiency limited bleomycin-induced fibrosis, bleomycin was administered to mice deficient in the gene for the Aα-chain of fibrinogen (*fib*). Contrary to our expectation, *fib*$^{-/-}$ mice developed pulmonary fibrosis to a degree similar to *fib*$^{+/-}$ littermate controls, which have a plasma fibrinogen level that is 70% of that of wild-type mice. Although elimination of fibrin from the lung was not in itself protective, the beneficial effect of PAI-1 deficiency was still associated with proteolytic activity of the plasminogen activation system. In particular, inhibition of plasmin activation and/or activity by tranexamic acid reversed both the accelerated fibrin clearance and the protective effect of PAI-1 deficiency. We conclude that protection from fibrosis by PAI-1 deficiency is dependent upon increased proteolytic activity of the plasminogen activation system; however, complete removal of fibrin is not sufficient to protect the lung.

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Introduction

Extravasation of plasma through damaged alveolar walls is a common occurrence during inflammatory lung diseases (1). Tissue factor that is present within the extravascular compartment triggers the coagulation cascade, leading to fibrin deposition (2). This fibrin matrix then serves as a scaffold onto which fibroblasts migrate and produce interstitial collagens. Although this fibrotic process is beneficial in circumstances such as the repair of cutaneous wounds, collagenous obliteration of airspaces renders the damaged areas of lung permanently nonfunctional. Therefore, successful repair of the injured lung requires timely removal of the fibrin-based matrix. This scenario is also presumed to be important in fibrotic processes that occur in other organs after tissue injury.

Recent experimental work using the bleomycin model of lung injury has supported the importance of the plasminogen activation system in modulating the development of pulmonary fibrosis. When bleomycin is administered to experimental animals, a multifocal inflammatory response occurs that progresses to fibrosis (3). This experimental model has served well for studying generic processes that occur when inflamed tissues are unable to effectively repair themselves and are replaced by collagenous scars. Using the bleomycin model, we have previously found that transgenic mice with a targeted deletion of their plasminogen activator inhibitor-1 (PAI-1) gene (*PAI-1*$^{-/-}$ mice) develop less pulmonary fibrosis than do wild-type mice (4). Furthermore, intratracheal instillation of an adenoviral vector containing a urokinase-type plasminogen activator (uPA) gene reduces the amount of collagen that accumulates after bleomycin exposure in mice (5).

A leading hypothesis for the protective role of the plasminogen activation system in pulmonary fibrosis has been that it is due to the plasmin-mediated clearance of fibrin. However, results of recent studies have suggested that other activities of the plasminogen activation system could theoretically influence lung injury and repair (reviewed in refs. 6–9). For example, plasmin may contribute significantly to the proteolytic activation of growth factors, cytokines, and matrix metalloproteinases, the degradation of matrix glycoproteins, and the clearance of necrotic tissue. In addition, specific components of the plasminogen activation system may have significant biological roles aside from control of plasmin-mediated proteolysis. In this regard, the

urokinase receptor may influence cell adhesion, cell migration, and the initiation of intracellular signaling events. Finally, plasminogen might also influence the repair process by serving as the precursor of angiostatin (10). Determining whether the critical function of the plasminogen activation system in pulmonary fibrosis is plasmin-mediated fibrin clearance or another of its many functions will assist in developing therapeutic interventions. In this study, we evaluate whether the protection caused by PAI-1 deficiency works through a plasmin-dependent proteolytic mechanism. We also evaluate the role of fibrin by determining whether its presence is required for pulmonary fibrosis to occur.

Methods

Materials. Chloramine-T, Ehrlich's solution, collagenase, DNase, Evans blue dye, formamide, heparin, human plasmin, and tranexamic acid were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Human fibrinogen was obtained from Calbiochem-Novabiochem (La Jolla, California, USA). Human Glu-plasminogen and human thrombin were purchased from American Diagnostica Inc. (Greenwich, Connecticut, USA). Texas red-conjugated BSA was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Goat anti-mouse fibrinogen antibody was purchased from Nordic Immunological Laboratories BV (Tilburg, The Netherlands). The VECTASTAIN Elite ABC kit was obtained from Vector Laboratories Inc. (Burlingame, California, USA).

Animals. *PAI-1*$^{-/-}$ mice (11) were originally provided by P. Carmeliet (University of Leuven, Belgium). These were subsequently backcrossed to C57BL/6 mice for eight generations. Wild-type C57BL/6 mice (*PAI-1*$^{+/+}$) were purchased from Charles River Laboratories Inc. (Wilmington, Massachusetts, USA). Aα-chain fibrinogen-deficient (*fib*$^{-/-}$) mice (12) and fibrinogen-heterozygotic (*fib*$^{+/-}$) mice were produced by mating C57BL/6 *fib*$^{-/-}$ males and *fib*$^{+/-}$ females that were inbred for six generations.

Bleomycin exposure. For each experiment, age- and weight-matched groups of mice were used. Mice were anesthetized with either intraperitoneal pentobarbital or inhalation of 2% isoflurane, and the trachea was exposed by a cervical incision. Bleomycin (Nippon Kayaku Co., Tokyo, Japan) was dissolved in PBS and then instilled intratracheally using a 27-gauge needle. The dose for *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice (average weight 20 ± 2 g) was 2.5 U/kg. A higher dose of bleomycin (5 U/kg) was given in experiments using *fib*$^{-/-}$ and *fib*$^{+/-}$ mice (average weight 27 ± 4 g) after preliminary experiments using 2.5 U/kg failed to produce consistent pulmonary fibrosis in the *fib*$^{+/-}$ mice.

Hydroxyproline assay. Hydroxyproline content in whole mouse lungs was used to quantify lung collagen content and was measured colorimetrically by a method described previously, with modifications (13). At the time of sacrifice, both lungs were removed and the extrapulmonary airways and blood vessels were excised and discarded. The lung parenchyma was homogenized in 1.0 ml of PBS, after which 1.0 ml of 12 N HCl was added, and the samples were hydrolyzed at 110°C for 24 hours. Five microliters of each sample was mixed with 5 μl of citrate-acetate buffer (5% citric acid, 1.2% glacial acetic acid, 7.25% sodium acetate, and 3.4% sodium hydroxide). One hundred microliters of chloramine-T solution (1.4% chloramine-T, 10% *N*-propanol, and 80% citrate-acetate buffer) was added, and the mixture was incubated for 20 minutes at room temperature. Ehrlich's solution was added and the samples were incubated at 65°C for 18 minutes. Absorbance was measured at 550 nm. Standard curves were generated for each experiment using reagent hydroxyproline as a standard. Results were expressed as micrograms of hydroxyproline contained in total lung tissue.

Inflammatory leukocytes in bronchoalveolar lavage fluid and collagenase digests of lung tissue. Using a modification of a previously described method (14), mice were sacrificed with a lethal dose of pentobarbital, the trachea was cannulated with an 18-gauge needle, and the lungs were lavaged twice with PBS. The lavage fluid was pooled and centrifuged, and the cell pellet was resuspended in erythrocyte-lysing buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.0372% Na_2EDTA, pH 7.4). The lavage cells were then washed with HBSS and resuspended in RPMI 1640. Immediately after lavage, the lung vascular bed was perfused with PBS, and the lungs were excised, minced, and digested enzymatically with digestion solution (RPMI 1640, 1 mg/ml collagenase, and 30 U/ml DNase) at 37°C for 30 minutes. The suspension was dispersed by repeated aspiration through a 10-ml syringe, and erythrocytes were lysed by suspending in erythrocyte-lysis buffer. The cells were then washed twice with HBSS, resuspended in RPMI 1640, and filtered through a 100-μm nylon mesh. Lung leukocytes were then purified by spinning through a 20% discontinuous Percoll gradient. Aliquots of cells from bronchoalveolar lavage fluid (BALF) and lung tissue digests were stained with trypan blue, and the cells were counted using a hemocytometer. Cells were spun onto glass slides using a cytocentrifuge (Shandon Inc., Pittsburgh, Pennsylvania, USA) and were stained with Diff-Quick (Fisher Scientific International, Pittsburgh, Pennsylvania, USA). Three hundred cells were examined on each slide and the percent of each leukocyte type was recorded.

Measurement of fibrinolysis within lungs. Fibrinolysis in murine lungs was measured by a method described previously, with modifications (15). Mice were sacrificed and 1 ml of DMEM containing fibrinogen (1.5 mg/ml), fluorescein-labeled fibrinogen (0.1 mg/ml), plasminogen (60 μg/ml), thrombin (0.2 U/ml), and Texas red-conjugated BSA (0.25 mg/ml) was injected intratracheally. The Texas red-conjugated BSA was included in the instilled fluid to allow for correction for any differences in dilution that may have occurred between samples during subsequent tissue processing. After the instillation, the trachea of each animal was ligated, and

the lungs were placed into a 50-ml tube and incubated at 37°C for 5 hours. The lungs were then minced, centrifuged at 10,000 *g* for 10 minutes, and the fluorescein and Texas-red levels in 20 μl of supernatant were determined. To correct for differences in dilution, the level of fluorescence from fluorescein was divided by the level of fluorescence from Texas red. During each experiment, separate aliquots of instilled solution were placed into plastic tubes. The fibrin matrices in one set of tubes were allowed to remain undisturbed (0% lysis control), and those in another set of tubes were lysed with excess plasmin (100% lysis control). Both sets of tubes were centrifuged, and the ratios of fluorescein to Texas-red fluorescence in the supernatants were determined. The percent of fibrin that was degraded in the lung was calculated assuming a linear relationship between the values of the 0% and 100% lysis controls.

Measurement of fibrin content of lung tissue. Fibrin deposited in murine lungs was measured by immunoblotting to detect an 80-kDa γ-γ chain dimer fragment that was generated by terminal digestion of fibrin with reagent plasmin (16). After intravenous injection of heparin (1 U per mouse) to limit postmortem clotting, mice were sacrificed and the lung vascular bed was perfused with PBS containing 500 U/ml heparin. The lungs were excised, placed in iced buffer (0.05 M Tris, 0.15 M NaCl, and 500 U/ml heparin, pH 7.6), and homogenized. Human plasmin prepared in the same buffer was added to a final concentration of 0.32 U/ml, and the mixture was incubated at 37°C for 8 hours with agitation. The plasmin-digested lungs were centrifuged and the supernatant was recovered. As a positive control, mouse plasma was obtained, clotted with thrombin, and digested with human plasmin at 37°C for 8 hours. The plasmin-digested clot was centrifuged and the supernatant was collected. Equal volumes of lung supernatants were boiled under reducing conditions in SDS sample buffer and then separated by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were electroblotted onto a nylon membrane, and the membranes were reacted with a goat anti-mouse fibrinogen antibody. The transfer membranes were then stained using the VECTASTAIN Elite ABC kit following the manufacturer's suggested protocol.

Measurement of pulmonary vascular permeability. Using a modification of a previously described method (17), Evans blue dye prepared in PBS was injected into the tail veins of mice at 20 mg/kg body weight. Mice were sacrificed after 1 hour, and serum samples were prepared from blood aspirated by cardiac puncture. After the lung vascular bed was perfused with PBS, the lungs were excised, placed in 2 ml of formamide, and homogenized. The homogenized lungs were incubated at 60°C for 16 hours, followed by centrifugation and collection of the supernatant. The absorbance of the supernatants and of serum samples diluted 50 times was measured on a spectrophotometer at 620 nm. A permeability index was calculated as the ratio of the absorbance of the lung supernatant to that of the serum.

Tranexamic acid treatment. To block the lysine binding sites on plasminogen and plasmin, tranexamic acid was administered by continuous subcutaneous infusion via osmotic pumps (model 2002; ALZA Corp., Palo Alto, California, USA) implanted under the dorsal skin of mice. The pumps were loaded to deliver 1.8 mg of subcutaneous tranexamic acid per day. In addition, tranexamic acid was added to the drinking water at 20 mg/ml to supplement the parenteral dose (18).

Histology. Lungs were inflation-fixed with 10% neutral-buffered formalin, and the trachea was ligated. Heart and lungs were removed and fixed, en bloc, in paraffin. Four-micron sections were prepared from the blocks and stained with hematoxylin and eosin or Gomori's trichrome to detect collagen.

Immunohistochemistry. Sections were stained immunohistochemically for fibrin(ogen) with polyclonal rabbit anti-mouse fibrinogen antibodies (19), and for fibronectin with rabbit anti-human fibronectin (DAKO Corp., Carpinteria, California, USA). Bound antibody was detected using biotinylated goat anti-rabbit immunoglobulin and the VECTASTAIN Elite ABC kit (both from Vector Laboratories Inc.) using diaminobenzidine as a peroxidase substrate (Sigma Chemical Co.). Sections were counterstained with hematoxylin.

Statistics. Results are expressed as mean ± SEM. Differences between treatment groups were analyzed using ANOVA with Fisher's PLSD test for pairwise comparisons (StatView; Abacus Concepts Inc., Berkeley, California, USA). A P value of less than 0.05 was considered statistically significant.

Results

Kinetics of collagen accumulation in bleomycin-injured lungs. Eitzman and colleagues have reported that *PAI-1*$^{-/-}$ mice developed less pulmonary fibrosis than did *PAI-1*$^{+/+}$ animals after bleomycin administration (4). To determine the kinetics of collagen accumulation, 2.5 U/kg body weight of bleomycin was instilled intratracheally into *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice. By 14 days after bleomycin treatment, the lung hydroxyproline content of *PAI-1*$^{+/+}$ mice increased ($P < 0.006$) above that of control animals receiving PBS alone, and continued to increase throughout the duration of the experiment (Figure 1a). The hydroxyproline content of lungs from *PAI-1*$^{-/-}$ mice receiving bleomycin increased more slowly, becoming significantly elevated above control levels on day 21 ($P < 0.01$), when it reached a plateau. At all measured time points after bleomycin delivery, the lung hydroxyproline content of *PAI-1*$^{-/-}$ mice remained below that of *PAI-1*$^{+/+}$ mice ($P < 0.04$). Lung hydroxyproline content of mice 28 days after receiving PBS was used as the control condition in these experiments, because we have previously found that the lung hydroxyproline content after administration of PBS is not different from that of mice receiving no manipulations (data not shown).

Survival of mice after bleomycin treatment. Twenty *PAI-1*$^{-/-}$ mice and twenty *PAI-1*$^{+/+}$ mice were given 2.5 U/kg per body weight bleomycin intratracheally. After 28

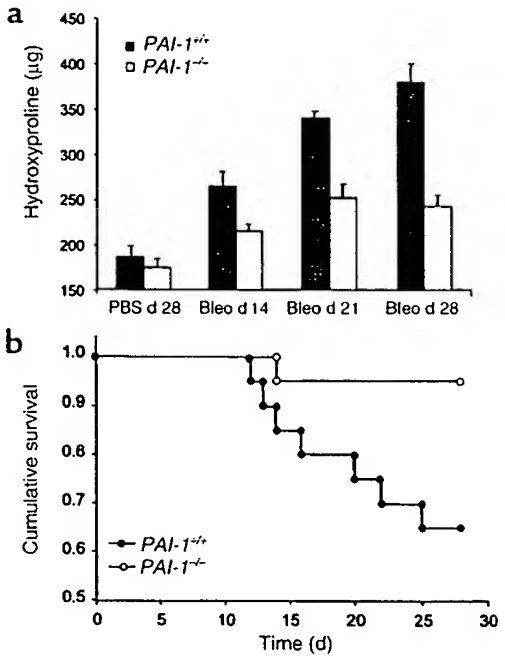

Figure 1
Effect of bleomycin on lung hydroxyproline content and survival in *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice. Bleomycin (2.5 U/kg) or PBS was instilled intratracheally into *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice. (a) On days 14, 21, and 28 after administration, lung hydroxyproline was measured. Data are expressed as mean ± SEM; n = 9–11 mice per group. (b) Kaplan-Meier survival curve for bleomycin-exposed *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice (n = 20 per group). Bleo, bleomycin.

days, when the experiment was terminated, one of the *PAI-1*$^{-/-}$ mice and seven of the *PAI-1*$^{+/+}$ mice had died (Figure 1b). The Mantel-Cox log rank test showed that the survival rate of the *PAI-1*$^{-/-}$ mice was significantly better than that of the *PAI-1*$^{+/+}$ mice ($P < 0.02$).

Lung inflammation after bleomycin injury. It is possible that the reduced collagen content of bleomycin-injured lungs in *PAI-1*$^{-/-}$ mice was due to an effect of PAI-1 deficiency on the development of the inflammatory process. To evaluate this possibility, the inflammatory cell contents of BALF and collagenase-digested lung tissue were analyzed. Seven days after bleomycin delivery, when the inflammatory response is known to be maximal (20), the total number of leukocytes in the BALF and collagenase digests of lung tissue had increased equally in *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice (Table 1). The increase in percent neutrophils and decrease in percent macrophages within BALF were also similar in the two genotypes. The percent lymphocytes and eosinophils remained unchanged for both genotypes. The relative distribution of inflammatory cell types in collagenase digests of lung tissue was unchanged by bleomycin in both *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ animals. These quantitative assessments of leukocyte accumulation were consistent with the visual appearance of the inflammatory infiltrates seen in histological sections of lung tissue (data not shown).

Fibrin content of lungs from bleomycin-injured mice. To determine if PAI-1 deficiency decreased fibrin accumulation after lung injury, the amount of fibrin contained in lung tissue from *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice was measured 7 days and 14 days after bleomycin administration. Lung tissue was homogenized and then digested by adding excess plasmin, and the fibrin content was assessed by the intensity of the plasmin-degraded γ-γ dimer fragment band seen at 80 kDa on immunoblots (21) (Figure 2a). As a positive control, fibrin formed from thrombin-treated mouse plasma was digested with plasmin and analyzed as above. The negative control sample was mouse plasma anticoagulated with ETDA. The lung digests of PBS-treated *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice showed no γ-γ dimer fragment. On day 4 after bleomycin, the γ-γ dimer fragment was easily detectable in the lungs of *PAI-1*$^{+/+}$ mice. The intensity of the γ-γ dimer fragment band increased further, reaching a plateau on day 7 and remaining elevated on days 14 and 21. In contrast, the intensity of the γ-γ dimer fragment band from bleomycin-exposed *PAI-1*$^{-/-}$ mice was less than that from *PAI-1*$^{+/+}$ mice on days 4 and 7, and had decreased further by days 14 and 21. At the level of sensitivity used to detect the plasmin cleavage products of γ-γ dimers, none were detected in lung homogenates in the absence of in vitro plasmin digestion.

Table 1
Bleomycin-induced accumulation of inflammatory cells within bronchoalveolar lavage fluid and lung tissue of *PAI-1*$^{+/+}$ and *PAI-1*$^{-/-}$ mice

	Genotype	Bleomycin	Total cells $\times 10^5$	Mac %	PMN %	Lymph %	Eos %
BALF	*PAI-1*$^{+/+}$	–	0.29 ± 0.03	97.3 ± 0.2	1.1 ± 0.4	1.3 ± 0.03	0.2 ± 0.1
		+	3.10 ± 1.01	81.4 ± 1.9	12.3 ± 1.3	4.6 ± 1.6	1.7 ± 1.0
	PAI-1$^{-/-}$	–	0.43 ± 0.09	97.2 ± 0.2	1.0 ± 0.2	1.9 ± 0.51	0.0 ± 0.0
		+	3.21 ± 0.47	81.2 ± 2.5	13.4 ± 1.6	2.6 ± 1.2	2.8 ± 1.3
Lung digest	*PAI-1*$^{+/+}$	–	14.7 ± 2.2	71.6 ± 4.4	12.8 ± 6.2	15.2 ± 3.6	0.4 ± 1.0
		+	27.0 ± 1.8	75.1 ± 1.7	9.8 ± 1.4	13.9 ± 3.8	1.2 ± 1.0
	PAI-1$^{-/-}$	–	17.9 ± 1.6	71.6 ± 2.2	10.2 ± 2.8	17.6 ± 2.5	0.7 ± 0.2
		+	26.8 ± 2.0	75.1 ± 1.6	11.6 ± 0.2	12.3 ± 1.3	0.9 ± 0.04

Bleomycin (2.5 U/kg) was administered intratracheally to *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice. On day 7, animals were sacrificed and the lungs were lavaged with PBS. Minced lung tissue was digested with collagenase, and leukocytes were separated from residual debris by filtration and Percoll-gradient centrifugation. The leukocyte numbers and differential counts (Mac, macrophages; PMN, neutrophils; Lymph, lymphocytes; Eos, esosinophils) were determined by hemocytometer and examination of cytospin preparations, respectively. Data presented as mean ± SEM; n = 4.

Lung permeability in bleomycin-exposed mice. The decreased amount of fibrin within the lungs of bleomycin-exposed *PAI-1*$^{-/-}$ mice could be due to either decreased formation of fibrin or its accelerated removal. Because fibrin is formed predominantly from the extravasation of plasma from damaged blood vessels, the effect of PAI-1 deficiency on lung vascular permeability that follows bleomycin administration was assessed. To measure permeability, mice were injected intravenously with Evans blue dye, which binds rapidly to circulating albumin. The accumulation of dye within lung tissue can be detected spectrophotometrically, thereby allowing measurement of macromolecular permeability. As shown in Figure 2b, the amount of vascular permeability 7 days after bleomycin administration was similar in *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice. Of note, Figure 2a shows that fibrin accumulation on day 7 was less in *PAI-1*$^{-/-}$ mice than in *PAI-1*$^{+/+}$ mice at a time when vascular permeability was similar. Thus, *PAI-1*$^{-/-}$ mice appear to be able to remove lung fibrin more efficiently than *PAI-1*$^{+/+}$ mice do. On day 14, less dye had accumulated in the lungs of *PAI-1*$^{-/-}$ mice than in lungs of *PAI-1*$^{+/+}$ mice. Hence, the reduced fibrin accumulation in *PAI-1*$^{-/-}$ mice on day 14 could be due to a combination of increased fibrin clearance and/or decreased vascular permeability.

Rate of fibrin clearance from mouse lungs. To assess the capacity of the lungs to degrade fibrin within the airspaces, an in situ fibrinolytic assay was used. Plasminogen and thrombin were added to fluorescein-labeled fibrinogen, and the mixture was immediately instilled into the lungs of mice ex vivo. After 5 hours, fibrinolysis was measured by determining the percentage of soluble fluorescent material that was soluble from lung minces. Experiments were performed on days 7 and 14 after bleomycin administration. Day-to-day variation in the percent fibrinolysis occurring in control mouse lungs prevented direct comparison of absolute values from experiments performed on different days. Nevertheless, on both day 7 and day 14, bleomycin-injured *PAI-1*$^{-/-}$ mice lysed fibrin more efficiently than did *PAI-1*$^{+/+}$ mice ($P < 0.002$; Figure 2c). Unexpectedly, fibrin degradation in *PAI-1*$^{+/+}$ mice was similar between those treated with PBS alone and those that were exposed to bleomycin. This is in contrast to studies of BALF where soluble fibrinolytic activity has been reported to be partially suppressed during pulmonary inflammation (22, 23).

Inhibition of plasmin activity with tranexamic acid. PAI-1 has multiple functions beyond inhibiting the enzymatic activity of plasminogen activators (6–9). It affects cellular adhesion and migration by interfering with the interaction between the uPA receptor and vitronectin. PAI-1 also initiates cellular uptake of the uPA receptor-uPA complex. It is possible that one or more of these other functions of PAI-1 could be instrumental in modulating the extent of fibrosis that occurs after lung injury. To determine if PAI-1 deficiency limits collagen deposition by allowing more plasmin activation to

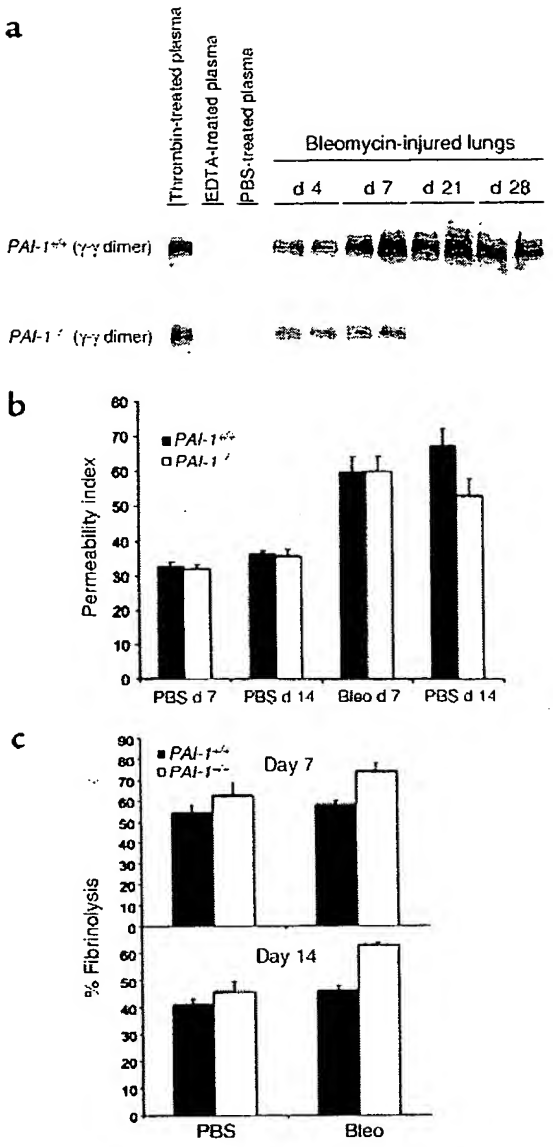

Figure 2
Dynamics of fibrin accumulation in bleomycin-exposed mice. Bleomycin (2.5 U/kg) or PBS was instilled intratracheally into *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice. (a) To measure tissue fibrin content, lungs were harvested from heparinized mice on the days indicated and were processed as described in Methods. As positive and negative controls, fibrin samples formed within thrombin-treated plasma and anticoagulated plasma were prepared and processed in an identical manner. Samples were separated by SDS-PAGE and then immunoblotted to detect plasmin-generated, fibrin-derived γ-γ dimer fragment. (b) Vascular permeability was measured 7 days and 14 days after bleomycin administration, using lung accumulation of Evans blue dye that was injected intravenously 1 hour before sacrifice. Data are expressed as mean ± SEM; $n = 7$ for bleomycin-injured mice, $n = 3$ for PBS control mice. (c) The rate of fibrin degradation was measured in lungs of *PAI-1*$^{-/-}$ and *PAI-1*$^{+/+}$ mice 7 days and 14 days after intratracheal instillation of PBS or bleomycin. At the time of sacrifice, fibrin was formed within pulmonary airspaces by intratracheally instilling fluorescein-labeled fibrinogen, plasminogen, and thrombin. After 5 hours, the percent of soluble fluorescent material was measured. Data are expressed as mean ± SEM; $n = 4$–7 mice per group.

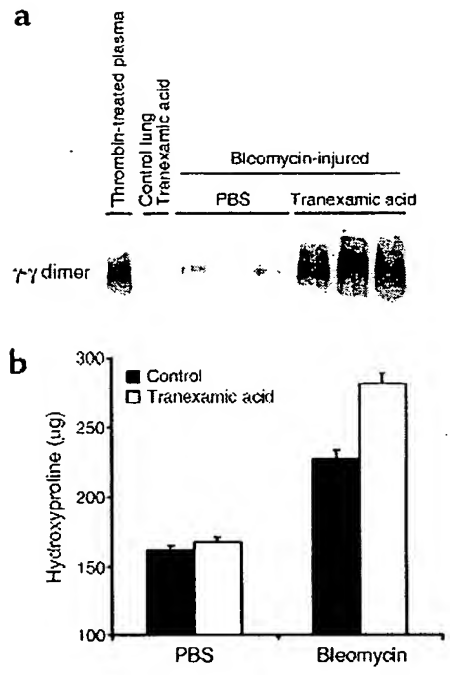

Figure 3
Effects of tranexamic acid on lungs of bleomycin-injured *PAI-1*$^{-/-}$ mice. PBS or bleomycin was instilled intratracheally into *PAI-1*$^{-/-}$ mice, and PBS or tranexamic acid was administered by subcutaneous osmotic pump and addition to drinking water. After 14 days, lungs were harvested and analyzed. (a) Immunoblot of fibrin-derived γ-γ dimer fragment in the plasmin-digested lungs of tranexamic acid-treated *PAI-1*$^{-/-}$ mice. (b) Hydroxyproline content of lung tissue. Results are expressed as mean ± SEM; n = 5-8 mice per bleomycin-treated group.

occur, we reversed this effect by administering tranexamic acid to *PAI-1*$^{-/-}$ mice. Tranexamic acid inhibits plasmin proteolysis both by blocking plasmin(ogen) binding to its proteolytic substrates (24, 25) and by interfering with plasminogen binding to cellular receptors, where cell surface-bound uPA efficiently converts it to plasmin (26). Administration of tranexamic acid to *PAI-1*$^{-/-}$ mice successfully reduced the plasmin activity in bleomycin-injured lungs, as indicated by an increase in lung fibrin content (Figure 3a). In the absence of bleomycin, fibrin did not accumulate within the lungs of mice receiving tranexamic acid. Importantly, this inhibition of plasmin activity by tranexamic acid caused a significant increase in lung collagen as measured by hydroxyproline content (P < 0.002; Figure 3b). These results support the importance of plasmin enzymatic activity in limiting lung fibrosis after an inflammatory injury. In separate experiments discussed below, tranexamic acid was administered to bleomycin-treated animals having wild-type *PAI-1* genes. No significant tranexamic acid effect was seen, indicating that normal PAI-1 expression masks any additional inhibitory effect of tranexamic acid.

Bleomycin-induced pulmonary fibrosis in mice that are genetically deficient in fibrinogen. The above results showed that PAI-1 deficiency enhances fibrinolytic activity and limits both fibrin accumulation and collagen deposition after bleomycin administration. To determine whether fibrin accumulation is a required step for bleomycin-induced fibrosis to occur, bleomycin was given to mice that were genetically deficient in fibrinogen (*fib*$^{-/-}$ mice). These animals, having a targeted deletion within the gene coding for the Aα chain of fibrinogen, have no circulating fibrinogen, whereas heterozygous animals (*fib*$^{+/-}$) have a plasma fibrinogen level that is 70% that of the wild type. (Note: Fibrinogen levels of *fib*$^{+/-}$ mice are reduced by less than half of wild-type levels because the synthesis of the Bβ chain appears to limit fibrinogen production in wild-type animals.) Contrary to our hypothesis that fibrinogen deficiency would protect from fibrosis, bleomycin caused an increase in lung hydroxyproline content in both *fib*$^{+/-}$ and *fib*$^{-/-}$ mice (P < 0.004; Figure 4).

The similarity in lung hydroxyproline content of *fib*$^{-/-}$ and *fib*$^{+/-}$ mice did not appear to be a consequence of selective death of mice of any one genotype after bleomycin instillation. A higher fraction of *fib*$^{-/-}$ mice did die in the first two days after surgery (~17% postsurgical mortality with either bleomycin or saline instillation) than *fib*$^{+/-}$ mice, which almost always survived the surgical trauma. Of those surviving the initial days after surgery, approximately 80% of both *fib*$^{-/-}$ and *fib*$^{+/-}$ mice survived to the day of lung collection. All mice lost 5-10% of body weight after the administration of bleomycin. Recovery of some weight occurred between 14 and 24 days, with no difference between *fib*$^{-/-}$ and *fib*$^{+/-}$ mice. PBS-treated mice in both groups also lost some weight initially after surgery, but regained weight at a faster rate than did bleomycin-treated mice (P < 0.007).

At 14 days and 24 days after bleomycin treatment, focal fibrotic lesions were seen in lungs of both *fib*$^{+/-}$ and *fib*$^{-/-}$ mice (Figure 5), whereas no lesions were observed in lungs collected from saline-treated animals of either genotype. Most of the lesions con-

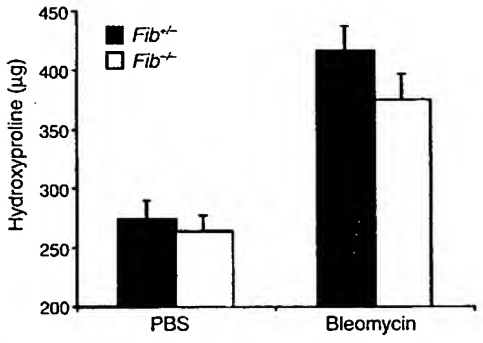

Figure 4
Effect of bleomycin (5 U/kg) on lung hydroxyproline content of *fib*$^{-/-}$ and *fib*$^{+/-}$ mice. Bleomycin (*fib*$^{+/-}$, n = 6; *fib*$^{-/-}$, n = 5) or PBS (*fib*$^{+/-}$, n = 6; *fib*$^{-/-}$, n = 3) was instilled intratracheally into *fib*$^{-/-}$ and *fib*$^{+/-}$ mice. Twenty-four days after administration, lung hydroxyproline was measured. Data are expressed as mean ± SEM.

tained some areas of fibrillar material that stained blue for collagen deposits with Gomori's trichrome stain, and were frequently associated with inflammatory cell accumulations consisting of lymphocytes and macrophages. The number of spindle-shaped cells resembling fibroblasts appeared to be increased in lesions from both genotypes. Other than the distinct absence of detectable fibrin(ogen) in lungs of $fib^{-/-}$ mice (Figure 5), no qualitative differences were seen in lesion composition or occurrence in either genotype. Immunohistochemical analyses for fibrin(ogen) in $fib^{+/-}$ mice revealed intense staining, presumably of fibrin, within lesions in which the normal alveolar architecture had been obliterated.

Immunohistology was also used to assess the distribution of fibronectin within lung tissue after bleomycin treatment. Fibronectin was chosen for study because it has cell-adhesive properties that might compensate for the absence of provisional fibrin matrices in $fib^{-/-}$ mice. Furthermore, fibronectin might accumulate in $fib^{-/-}$ animals if the absence of fibrinogen were to cause increased leaking of plasma proteins after lung injury. The intensity and pattern of fibronectin immunostaining were both indistinguishable between $fib^{+/-}$ and $fib^{-/-}$ mice (Figure 5). Fibronectin was weakly detectable within the interstitium of normal-appearing lung tissue. In contrast, prominent fibrillar staining was seen within the fibrotic lesions of $fib^{+/-}$ and $fib^{-/-}$ mice. A similar pattern was seen in wild-type and $PAI\text{-}1^{-/-}$ mice after bleomycin administration (data not shown).

The effect of tranexamic acid on $fib^{-/-}$ mice was also studied using the same drug delivery protocol used for the $PAI\text{-}1^{-/-}$ mice. Contrary to what was seen in bleomycin-treated $PAI\text{-}1^{-/-}$ mice, tranexamic acid in $fib^{-/-}$ mice caused only a minor, statistically insignificant increase in lung hydroxyproline content 14 days after bleomycin administration (Figure 6). However, tranexamic acid also failed to cause a significant increase in hydroxyproline when given to bleomycin-treated $fib^{+/-}$ mice. Apparently, in both $fib^{-/-}$ and $fib^{+/-}$ mice, tranexamic acid did not add substantially to the inhibitory effect that is conferred by normal PAI-1 expression.

Discussion

A relationship between the plasminogen activation system and the development of pulmonary fibrosis after lung inflammation is firmly established. BALF from patients with acute respiratory distress syndrome (27, 28) or a variety of chronic inflammatory lung diseases (22) show suppressed fibrinolytic activity, largely due to increases in fibrinolytic inhibitors. Mice with impaired fibrinolysis caused by either overexpression of a *PAI-1* transgene (4) or by targeted deletion of the plasminogen gene (29) develop increased lung fibrosis after lung inflammation. Conversely, increasing the activity of the fibrinolytic system by targeted deletion of *PAI-1* genes (as investigated in this report) protects the lung from fibrosis induced by inflammation. Furthermore, increasing uPA in the lung by direct protein administration (30) or by gene transfer (5) also reduces

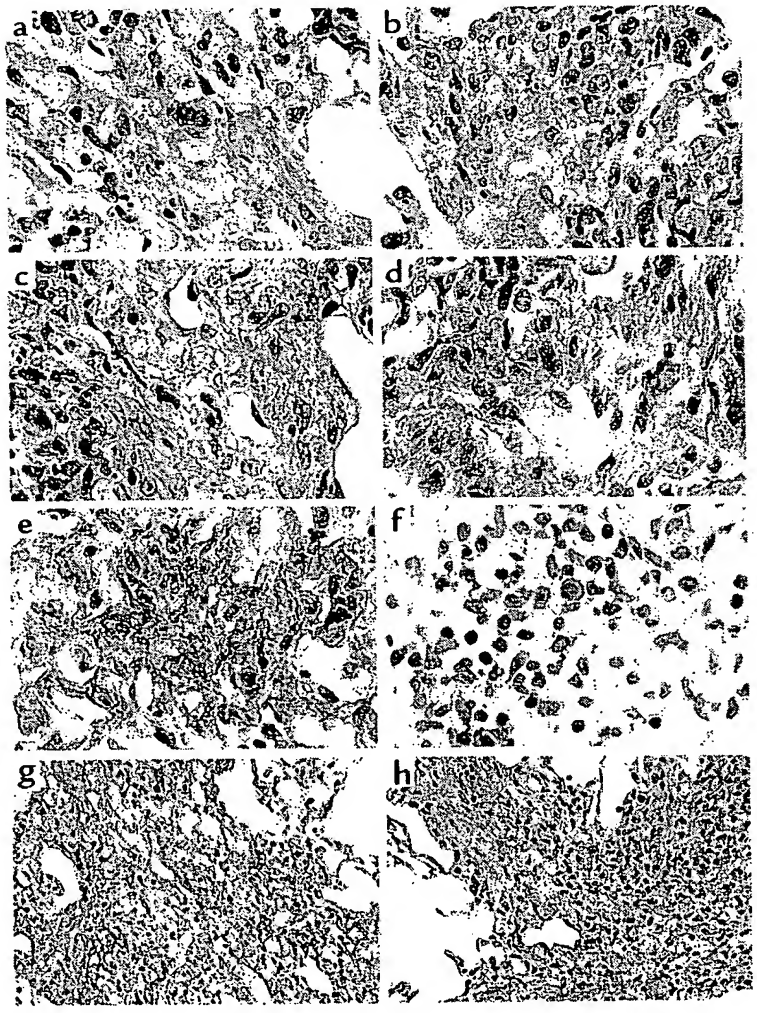

Figure 5
Microscopic appearance of focal fibrotic lesions in lungs of $fib^{+/-}$ and $fib^{-/-}$ mice treated with bleomycin. Fibrotic lesions with associated inflammatory infiltrates typical of those occurring in lungs of $fib^{+/-}$ (a) and $fib^{-/-}$ (b) mice 24 days after bleomycin instillation (hematoxylin and eosin staining). Gomori's trichrome stain illustrating collagen deposition within lesions of both $fib^{+/-}$ (c) and $fib^{-/-}$ (d) mice. Immunohistochemical stain showing that fibrin(ogen) is associated with lesions of $fib^{+/-}$ mice (e; brown reaction product) but is absent in $fib^{-/-}$ mice (f). Immunostaining for fibronectin shows a similar pattern in $fib^{+/-}$ (g) and $fib^{-/-}$ (h) mice.

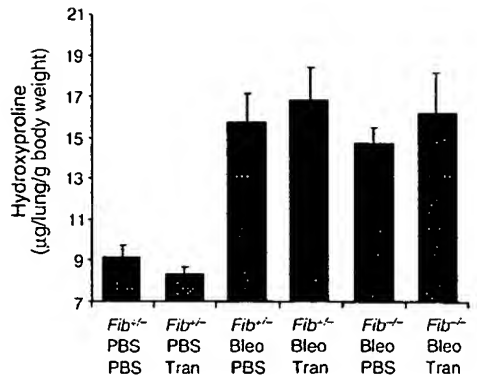

Figure 6
Effect of tranexamic acid on lung hydroxyproline content of bleomycin-treated lungs of *fib*$^{+/-}$ and *fib*$^{-/-}$ mice. PBS or bleomycin was instilled intratracheally, and PBS or tranexamic acid was administered by subcutaneous osmotic pump and addition to drinking water. After 14 days, lungs were harvested and analyzed for hydroxyproline content. Results expressed as mean ± SEM; n = 4–7 animals per group. Tran, tranexamic acid.

fibrosis. Importantly, these latter interventions can be delivered after the inflammatory injury has already begun, making the approach potentially useful as a treatment for pulmonary fibrotic diseases. To develop this theory, we studied the mechanisms by which *PAI-1*$^{-/-}$ mice limit the development of pulmonary fibrosis.

Our initial hypothesis was that accelerated fibrin clearance from the damaged lung would remove the provisional matrix onto which fibroblasts can migrate and form collagenous scars. In agreement with this hypothesis, fibrin was found to accumulate less in *PAI-1*$^{-/-}$ mice after lung injury than in wild-type mice. The reduction in fibrin accumulation did not appear to be due to reduced fibrin formation because the extent of inflammatory injury and permeability leak seen 7 days after bleomycin delivery was similar in the *PAI-1*$^{-/-}$ mice and the wild-type mice. Instead, the reduced fibrin accumulation in *PAI-1*$^{-/-}$ mice was probably a result of enhanced fibrinolysis, a phenomenon that was demonstrated directly by comparing the rate of degradation of fibrin matrices formed ex vivo in the lungs of *PAI-1*$^{-/-}$ and wild-type animals. In fact, the difference that was measured in the fibrinolytic rate of lungs from *PAI-1*$^{-/-}$ mice and wild-type mice is probably lower than the true difference in fibrinolytic capacity. The reason for the reduced difference is that the experimental matrix that was generated within the lungs was formed by instilling reagent fibrinogen, plasminogen, and thrombin. Such a matrix would minimize a major difference between wild-type and *PAI-1*$^{-/-}$ animals, namely, the presence of PAI-1 in endogenously formed matrices in wild-type mice and its absence in *PAI-1*$^{-/-}$ mice. The difference in fibrinolytic rates that we reported between the two genotypes thus depended upon the small amount of PAI-1 present in the alveolar lining fluid before the matrix-forming substrates were instilled. It is likely that the same considerations explain the similarity in fibrinolytic rates seen in wild-type mice treated with PBS or bleomycin. Forming the intra-alveolar matrices using reagent fibrinogen and thrombin would diminish the effect of increased PAI-1 production that is induced by bleomycin.

To our surprise, the experiments using *fib*$^{-/-}$ mice showed that fibrin was not required for fibrosis to occur in the injured lung. Despite the consistent presence of fibrin during inflammatory lung diseases and the ability of fibrin to serve as a provisional matrix for wound repair, fibrosis occurs in its absence. The minor difference in lung hydroxyproline content between the bleomycin-treated *fib*$^{+/-}$ and *fib*$^{-/-}$ mice was not statistically significant. In retrospect, previous observations of events occurring in nonpulmonary organs of *fib*$^{-/-}$ mice hinted at this possibility. In particular, scar tissue develops in *fib*$^{-/-}$ mice during the resolution of subcapsular hematomas that form spontaneously in the liver (12) and during the healing of full-thickness skin incisions (31). Nevertheless, the finding of pulmonary fibrosis in *fib*$^{-/-}$ mice does not exclude the possibility that excessive or prolonged fibrin deposition in the lung can contribute to fibrosis under specific conditions. For example, the increased fibrosis associated with plasminogen deficiency or inhibition of plasminogen activation could be, at least in part, driven by the cellular organization of fibrin deposits. If this theory is correct, then based on the studies presented here, genetically imposing fibrinogen deficiency in plasminogen-deficient mice would be expected to substantially reduce, but not eliminate, lung fibrosis. On the other hand, the further reduction in pulmonary fibrin that would be achieved by genetically imposing fibrinogen deficiency in PAI-1–deficient mice would probably result in little or no further reduction in fibrosis relative to mice lacking PAI-1 alone. Furthermore, the results of this study would predict that the relative resistance of PAI-1–deficient mice to fibrosis would be maintained regardless of the presence or absence of fibrinogen. Studies are underway to directly test these working hypotheses.

Although fibrin is obviously a key physiologic target of plasmin (31), biologically significant roles of plasmin beyond fibrin clearance have been documented in specific physiological and pathological contexts. An important physiological role of plasminogen activation in hepatic repair that is independent of plasmin-mediated fibrinolysis has been recently established (9). Plasminogen-deficient mice were shown to have a marked impairment in the reparative removal of necrotic liver tissue; this impediment persisted in the absence of fibrin deposition. A role of plasmin outside fibrinolysis has also been recognized in the pathological context of kainate-induced neurodegeneration in the hippocampus (32). Detailed studies revealed that laminin is probably one critical plasmin substrate relevant to neurodegeneration. Plasmin may also limit pulmonary fibrosis by con-

tributing to the clearance of common extracellular matrix components (reviewed in refs. 33 and 34). Alternatively, plasmin-mediated activation of protease zymogens, such as latent metalloproteinases (35), may limit pulmonary fibrosis by secondarily removing extracellular matrix proteins whose persistence would obliterate alveolar spaces. Although these proteolytic activities could theoretically cause harm by destroying normal tissue, their net effect appears beneficial during lung inflammation in *PAI-1*$^{-/-}$ mice. Additional targets of the plasminogen activation system that might be relevant to pulmonary fibrosis are latent growth factors (8). In particular, HGF is activated by plasminogen activators in vitro (36) and might promote alveolar epithelial cell proliferation and repair of damaged alveolar surfaces in vivo (37). Recent studies have shown that administration of HGF can reduce bleomycin-induced lung injury (38). Taken together, the fibrotic tendency may be modified by plasminogen activators/plasmin through combinatorial cleavage of many distinct substrates, including fibrin and nonfibrin substrates.

The beneficial effect of PAI-1 deficiency on pulmonary fibrosis could also be due to nonproteolytic features of the plasminogen activator/plasmin system. In vitro studies have shown that the binding of PAI-1 to the extracellular matrix protein vitronectin reduces the affinity of uPA receptor for vitronectin (39, 40). When uPA is bound to its cell-surface receptor, interaction with PAI-1 increases the removal of uPA receptor complexes from the cell surface (41). Among other activities, the uPA receptor is involved in initiating intracellular signaling (42). The net in vivo effects of these complex PAI-1 interactions are difficult to predict given their potential roles in adhesion, migration, and intracellular signaling involving leukocytes, fibroblasts, epithelial cells, and endothelial cells. However, even if these nonproteolytic features are operative in limiting pulmonary fibrosis, certain observations still point strongly to a proteolytic role for the plasminogen activator/plasmin system. In particular, tranexamic acid was found to reverse the beneficial effects of PAI-1 deficiency. Tranexamic acid inhibits plasmin-mediated proteolysis by interfering with the binding of plasmin to its target substrates (24), or by preventing plasminogen binding to cellular surfaces where it can be activated efficiently by receptor-bound uPA (43). Although tranexamic acid promoted fibrosis in animals deficient in PAI-1, similar effects were not seen in animals with a normal *PAI-1* genotype. Apparently, the regulatory effects of PAI-1 on plasminogen activation were not influenced significantly by the addition of tranexamic acid.

Modulation of fibrotic processes by the plasminogen activator/plasmin system is not unique to the lung. Decreased plasminogen activation was found to result in increased amounts of extracellular matrix within immune-damaged renal glomeruli (44) and synovial tissue (45), whereas increased plasminogen activation reduced the development of adhesions within an inflamed pleural space (46) and reduced capsular fibrosis of the optic lens after complicated cataract surgery (47). From these data, a pattern emerges in which impaired plasminogen activation in multiple organ systems is generally associated with increased fibrosis, whereas interventions that enhance plasminogen activator activity are associated with less fibrosis. However, there are situations in which a different relationship is seen. For example, after myocardial infarction, uPA-deficient mice were found to accumulate less collagen in the necrotic areas (48). Notably, this decreased deposition of extracellular matrix is associated with a severe impediment in the clearance of necrotic cardiomyocytes and diminished fibroblast infiltration. The seemingly contradictory profibrotic aspect of plasminogen activation in some tissues (e.g., ischemic heart tissue) and the antifibrotic potential of increased plasminogen activator activity in other tissues (e.g., bleomycin-challenged lung tissue) might be reconciled by distinct biological features associated with different sites and types of injury. Specific features that might determine the local pro- or antifibrotic consequences of defects in plasminogen activation include (1) the presence (e.g., ischemic tissue) or absence (e.g., skin incision) of dense fields of necrotic cells; (2) the composition of the extracellular matrix; and (3) the availability and type of other proteolytic systems for clearing matrix components and cellular debris.

In conclusion, our results show that PAI-1 deficiency reduces the extent of pulmonary fibrosis that follows inflammatory lung injury. The improvement tracks closely with the proteolytic activities of the plasminogen activator/plasmin system. Despite our supposition that accelerated removal of fibrin was responsible for limiting fibrosis, fibrin is not required for fibrosis to occur. Degradation of additional substrates or activation of other antifibrotic pathways may be involved in the beneficial effect of augmenting the plasminogen activation system.

Note added in proof. Subsequent to the submission of this article, a short communication was published reporting the development of bleomycin-induced fibrosis in mice with a targeted deletion of the fibrinogen γ-chain (49).

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REVIEW

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The bleomycin-induced scleroderma model: what have we learned for scleroderma pathogenesis?

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Abstract Scleroderma is a fibrotic condition characterized by immunologic abnormalities, vascular injury and increased accumulation of extracellular matrix (ECM) proteins in the skin. Although the etiology of scleroderma has not yet been fully elucidated, a growing body of evidence suggests that ECM overproduction by activated fibroblasts results from complex interactions among endothelial cells, lymphocytes, macrophages and fibroblasts, via a number of mediators, such as cytokines, chemokines and growth factors. For a better understanding of the pathophysiology of scleroderma, animal models are important tools. We established a murine model of cutaneous sclerosis by local treatment of bleomycin. This model reproduces several histological as well as biochemical aspects of human scleroderma. However, it must be emphasized that studying animal models cannot answer all the problems of human scleroderma. In this review, we introduce current insights into the pathogenesis of bleomycin-induced scleroderma, and discuss its contribution to our understanding of the pathogenesis of, and treatments for, human scleroderma.

Keywords Scleroderma · Cytokine · Bleomycin · Mouse model

Introduction

Systemic sclerosis (SSc) is a connective tissue disease which shows fibrosis of the skin and various internal organs [55, 63]. Although the pathogenesis of SSc has not been fully elucidated yet, it is characterized by the excessive accumulation of extracellular matrix (ECM) proteins in the skin and various internal organs, vascular injury and immunological abnormalities. In early stages of SSc, activated fibroblasts in the affected areas produce high amounts of collagen [68]. Histological analysis of the initial stage of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis [22], which is associated with increased collagen synthesis in the surrounding fibroblasts [102]. A number of studies have demonstrated the crucial role of several fibrogenic cytokines released from immunocytes in initiating the sequence of events leading to fibrosis.

Animal models are useful in providing clues for understanding various human diseases and for testing new methods of treatment. Although animal models that exhibit all the aspects of SSc are not currently available, several experimental animal models, such as tight skin (Tsk) mouse, Tsk2 mouse, UCD 200 chicken, bleomycin-induced murine scleroderma, sclerodermatous graft-vs-host disease (Scl-GvHD) mouse and exogenous injections of transforming growth factor-β (TGF-β)/connective tissue growth factor (CTGF)-induced murine fibrosis model, have been examined so far. In this review, we discuss recent insights gained from use of bleomycin-induced murine scleroderma.

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Bleomycin-induced scleroderma model

Bleomycin, originally isolated from the fungus *Streptomyces verticillus* [115], is a frequently used antitumor antibiotic effective against various kinds of cancers. Lung fibrosis is a well-known side effect of bleomycin, and bleomycin-induced pulmonary fibrosis is an established rodent model resembling human lung fibrosis. Rodents develop acute alveolitis, followed by intense interstitial inflammation when given bleomycin intratracheally. At later stages, ECM including collagen, fibronectin, hyaluronan and small proteoglycans were found to be increased in the lung [93, 119]. Mountz et al. [74] reported that rats injected repeatedly with sublethal doses of bleomycin over a 58-week period developed severe dermal fibrosis similar to those found in human

scleroderma, with structural abnormalities of collagen fibers.

We established the bleomycin-induced scleroderma model [122, 123, 127, 131, 135, 138]. Dermal sclerosis was induced by repeated subcutaneous injections of bleomycin into the shaved back skins. The methods are described in detail elsewhere [139]. Histopathological examination revealed definite dermal sclerosis characterized by thickened collagen bundles, and the deposition of homogenous materials in the thickened dermis with cellular infiltrates, which mimicked the histologic features of human scleroderma. Dermal thickness gradually increased, up to twofold compared with PBS injections, with the onset of the sclerosis. Mast cells increased in number in tandem with the induction of dermal sclerosis. Also, a marked degranulation was found in particular in the early phase, with elevated plasma histamine levels [122]. In some strains, epidermal thickness was also induced as well [127]. Further, lung fibrosis showing thickened alveolar walls with cellular infiltrates was also induced early on. However, the kidney, liver and heart were not involved.

Cutaneous changes were generally localized to the area surrounding the injected site, and sclerotic changes were not induced in the remote regions, such as fingers or abdominal skin. The induced sclerotic changes remained at least 6 weeks, after the stoppage of bleomycin injections. Thickness of vascular wall in the deep dermis was also observed. Dermal sclerosis was induced in various mice strains, although there was some variation among strains in the intensity of the symptoms and the period required to induce dermal sclerosis. C3H/He, DBA/2, B10.D2 and B10.A strains demonstrated intense dermal sclerosis by bleomycin treatment, suggestive of bleomycin-"susceptible" [83, 127]. Hydroxyproline contents in the bleomycin-treated skin were significantly increased in comparison with those of the PBS-treated skin. Increased production as well as upregulation of mRNA levels of type I collagen were evident in the sclerotic skin [122, 127]. Instrumental analysis also showed skin sclerosis objectively [138]. It is shown that scleroderma skin express α-smooth muscle actin (SMA) [101], and myofibroblasts are shown to persist in scleroderma fibroblasts cultures [52]. In the bleomycin-induced scleroderma, α-SMA-positive myofibroblasts were observed in the dermis, and gradually increased in tandem with the induction of dermal sclerosis [135]. Treatment with anti-TGF-β antibody reduced myofibroblast formation in the lesional skin [135].

Interestingly, autoantibodies were detected in the serum after bleomycin treatment [122]. SSc exhibits various types of autoantibodies, such as anti-topoisomerase-I and anti-centromere antibodies and less frequently antinucleolar antibodies. Anti-topoisomerase-I antibodies are associated with diffuse SSc, increased frequency of pulmonary fibrosis and higher mortality. The anti-polymyositis–scleroderma (PM–Scl) autoantibody is associated with the polymyositis/scleroderma overlap syndrome. Anti-Th/T0antibodies are associated with milder skin and systemic involvement but with more severe pulmonary fibrosis and overall worse prognosis. Anti-RNA-polymerase family antibodies and antifibrillarin antibodies are predictive of diffuse SSc with systemic involvement and greater mortality [9]. An analysis of the types of autoantibodies in the bleomycin model awaits future investigation.

Table 1 shows a summary comparison of this murine and human scleroderma. Bleomycin hydrolase inactivates bleomycin by hydrolyzing the amide bond in the β-aminoalanineamide moiety. Due to the lack or shortage of this enzyme in the lungs and the skin [84, 110], bleomycin-induced fibrosis and sclerosis occurs predominantly in these organs. The features which are speculated to contribute to the primary and secondary events in the bleomycin-induced scleroderma are shown in Table 2. Furthermore, factors suspected of having an important but as yet fully unspecified role are listed in Table 3.

Effect of bleomycin on different types of cells

T cells

Mononuclear cell infiltration in the skin is one of the most characteristic histological features in early scleroderma [22], which is suggested to cause secretion of cytokines that stimulate ECM production. Moreover, infiltrating T cells, predominately CD4+, are also the

Table 1 Characteristics of bleomycin-induced murine scleroderma

Features	Bleomycin-induced scleroderma	Human SSc
Scleroderma	+	+
Sclerodactyly	-	+
Epidermis		
Epidermal proliferation	+	-
Dermis		
Increased dermal thickness	+	+
Thickened collagen fibers	+	+
Increased collagen deposition	+	+
Thickened vascular wall	+	-
Mononuclear cell infiltrate	+	+
Mast cell increase	+	+
Increased procollagen gene expression	+	+
Increased TGF-β expression	+	+
Increased CCL2 expression	+	+
Myofibrotic phenotype changes	+	+
Enhanced apoptosis of mononuclear cells	+	?
Visceral involvement		
Pulmonary	+	+
Gastrointestinal	-	+
Vascular	-	+
Renal	-	+
Cardiac	-	+
Hepatic	-	+
Apoptosis	+	+
Autoantibody	+	+
Scl-70	?	+

Table 2 Skin features of suggested primary/secondary contributory factors in bleomycin-induced scleroderma

Primary	Secondary
Increased collagen deposition	Epidermal proliferation
Mononuclear cell infiltrates	Increased dermal thickness
Increased procollagen gene expression	Thickened collagen fibers
Increased TGF-β expression	Thickened vascular wall
Increased CCL2 expression	Mast cell increase
Myofibrotic phenotype changes	
Enhanced apoptosis of mononuclear cells	

Table 3 Possible contributory molecules

TGF-β
PDGF
IL-4
CCL2
HSP47
CTGF
TNF-α
IL-13
CD40

major lymphocytes seen in the involved skin of scleroderma. As in human SSc, T cells, macrophages and mast cells are present in increased numbers or in an activated state in involved tissues in animal models of SSc. In hereditary avian scleroderma, lymphocytes in the deep dermis and subcutaneous tissues are enriched for α/β, CD4+ T cells [29]. Chronic GvHD is induced in mice by T cell allorecognition of major or minor histocompatibility complex-encoded molecules [43]. CD4+ T cells have been shown to be required for the excessive accumulation of dermal collagen in Tsk mice [117]. A number of mononuclear cells, including T cells and macrophages, infiltrates into the lesional skin following bleomycin treatment [121]; however, dermal sclerosis can be induced even in severe combined immunodeficient (SCID) mice to a degree comparable to control mice [131], and nude mice as well [138], following bleomycin exposure.

Macrophages

Activated macrophages are among the first immune cells to increase in the early stages of fibrosis. These cells release a number of proinflammatory and fibrogenic mediators such as TGF-β and platelet-derived growth factor (PDGF) [53]. Early scleroderma skin contains an increased number of CD14-positive cells (monocytes/macrophages) compared with normal skin [54]. Ishikawa et al. [42] demonstrated that the ratio of infiltrating macrophages to T cells was high, suggesting an important role of cutaneous macrophages in scleroderma. Bleomycin was also shown to induce alveolar [13] as well as peripheral [124] macrophages to produce growth stimulatory factors for fibroblasts. In Scl-GvHD mice, a number of CD11b+2F8+ monocytes/macrophages and CD3+ T cells were seen in the skin [144]. In the bleomycin model, a number of macrophages were detected along with T cells [122], and were assumed to have an important role as a major source of a number of mediators.

Mast cells

Mast cells are suggested to be one of the important initiators of SSc, since they increase in the lesional skin during the early stages scleroderma [35, 79]. Mast cells produce a number of cytokines, growth factors and mediators that are capable of activating fibroblasts or endothelial cells. Potential fibrogenic factors, which are produced by mast cells include histamine, tryptase, TGF-β, basic fibroblast growth factor (bFGF), interleukin-4 (IL-4) and PDGF. On the other hand, TGF-β can induce mast cell migration [27, 82]. In Tsk mice, mast cells are abundant in the thickened dermis and exhibit prominent degranulation; conversely, it has been reported that a decrease in fibrosis is associated with inhibition of mast cell degranulation [116]. By contrast, pulmonary fibrosis can be induced by bleomycin in genetically mast cell-deficient mice [72]. In the bleomycin model, mast cells increased in number with degranulation, in tandem with the induction of dermal sclerosis [122]. On the contrary, bleomycin could induce dermal sclerosis even in genetically mast cell-deficient WBB6F1-W/Wv mice similarly to control littermates [123]. TGF-β can be detected immunohistologically in the infiltrating cells in both WBB6F1-+/+ and WBB6F1-W/Wv mice [123]. These TGF-β-positive cells are predominantly composed of macrophages. Mast cells may be associated with, but are not a prerequisite for the induction of dermal sclerosis, suggesting that mast cells may not be the sole pathway to the induction of sclerosis.

Eosinophils

Eosinophil infiltration is occasionally seen in association with skin fibrosis [30]. Infiltration of eosinophils precedes and parallels the development of the lung fibrosis induced by bleomycin [143]. A report gives evidence that eosinophils represent a primary cellular source of TGF-β [142]. In vitro studies have shown that eosinophils readily bind to fibroblasts, leading to the release of mitogens that augment fibroblast proliferation [90] and collagen production [6]. On the contrary, bleomycin-induced pulmonary fibrosis was independent of eosinophils [31]. IL-5 and eotaxin are important in the differentiation, proliferation, recruitment, activation and chemotaxis. Fibroblasts can be a source of eotaxin under appropriate cytokine stimulation [111]. A recent study demonstrates that bleomycin-induced pulmonary fibrosis can be induced both in IL-5 transgenic and deficient mice, suggesting that eosinophils and T cells contribute distinctly to the development of fibrosis via production of different cytokine components [37]. In the lesional skin of the bleomycin model, eosinophils increased in number [125].

Fibroblasts

Fibroblasts are stimulated by inflammatory cells, such as activated T cells, monocytes/macrophages, mast cells and eosinophils. Additionally, recent evidence has suggested that fibroblasts themselves are not only structural elements but also part of the immune system, and can be activated to display new functions important for controlling ECM synthesis and for producing various cytokines/growth factors, chemokines, growth factor receptors, integrins and oxidants. In vitro, bleomycin upregulates collagen mRNA expression in human lung and dermal fibroblasts [12, 128]. However, newly synthesized collagen is rapidly degraded in bleomycin-treated lung fibroblasts [108], suggesting that increased collagen degradation may be responsible for the remodeling process. Also, bleomycin upregulates TGF-β1 mRNA expression in cultured rat lung [12] and human skin fibroblasts [128]. Increased TGF-β mRNA transcription is followed by TGF-β mRNA accumulation and TGF-β protein, which is followed by increased procollagen gene transcription [7]. It was shown that TGF-β is a mediator of the fibrotic effect of bleomycin at the transcriptional level and that the TGF-β response element is required for bleomycin stimulation of the proα1(I) collagen promoter [51].

Endothelial cells

Endothelial cells have been reported to play an important role in the inflammatory as well as fibrotic process. In vitro studies showed a dose-dependent stimulation of endothelial cell secretion of collagen synthesis by bleomycin [87]. This stimulatory activity was inhibited by the anti-TGF-β antibody [87]. Recent research has shown that cellular adhesion molecules (CAMs) are involved between immune cells, fibroblasts, endothelial cells and ECM in the lesional skin of scleroderma. Dermal endothelial cells of SSc patients have increased expression of E-selectin [11], which is important in the homing of inflammatory cells to the skin. In vitro, bleomycin directly induces E-selectin expression in endothelial cells through activation and nuclear translocation of NF-κB/Rel [41]. In the bleomycin-treated lung tissues, E-selectin expression was upregulated [4]. CAMs are suspected of being responsible for the homing of pathologic inflammatory cells to the skin, and the adhesion step may be important to the development of the initial pathologic changes of bleomycin-induced scleroderma.

Role of cytokines in bleomycin-induced scleroderma

TGF-β

TGF-β plays a key role in the pathogenesis of fibrosis. TGF-β, which is found abundantly in platelets and released from activated macrophages or lymphocytes, is a strong chemoattractant for fibroblasts [95]. TGF-β increases the synthesis of ECM, such as collagen type I and type III, or fibronectin by fibroblasts, modulates cell-matrix adhesion protein receptors, and regulates the production of proteins that can modify the ECM by proteolytic action, such as plasminogen activator (PA), an inhibitor of plasminogen or procollagenase [95]. In addition, TGF-β is capable of stimulating its own synthesis by fibroblasts through autoinduction [80]. TGF-β induces rapid fibrosis and angiogenesis when injected subcutaneously into newborn mice [94]. Thus, maintenance of increased TGF-β production may lead to the progressive deposition of ECM, resulting in fibrosis. Actually, TGF-β mRNA is elevated in the lesional skin of SSc and other fibrotic conditions [28, 56, 86], and also shown to co-localize with type I collagen in scleroderma skin [56]. Blocking its bioactivity can suppress matrix production and modulate cutaneous fibrosis [70, 145]. In the bleomycin model, immunohistological analysis showed that TGF-β was detected on the infiltrating cells, which were predominantly composed of macrophages, as well as fibroblasts at sclerotic stages. TGF-β1 and -β2 mRNA expression was also detected in the lesional skin. Additionally, expression and synthesis of TGF-β1 were increased in bleomycin-"susceptible" mice strains [83].

Signaling by TGF-β elicits potent profibrotic responses in tissue fibroblasts and mesenchymal cells. Upon binding of TGF-β to the type II receptor, the type I receptor becomes activated, and signaling to the nucleus occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad family. Three families of Smads have been identified; receptor-regulated Smad2 and -3 (R-Smads), common partner Smad4 (Co-Smad) and inhibitory Smad6 and -7 (I-Smads). Smad7 has been shown to act as an intracellular antagonist of TGF-β signaling, and an inhibitor of TGF-β-induced transcriptional responses. An antifibrotic effect of Smad7 has recently been demonstrated in bleomycin-induced lung fibrosis [77]. In scleroderma skin and cultured scleroderma fibroblasts, basal level and TGF-β-inducible expression of Smad7 are selectively decreased, whereas Smad3 expression is increased [15]. On the other hand, Smad7 expression levels in scleroderma fibroblasts are unclear and disputed [2, 73]. In the bleomycin-treated skin, fibroblasts showed predominantly nuclear localization of Smad3 and intense staining for phospho-Smad2/3 [109]. On the other hand, expression of Smad7 was downregulated, which may account for sustained activation of TGF-β/Smad signaling in the lesional skin. Targeted disruption of Smad3 ameliorated bleomycin-induced scleroderma, unassociated with inflammation [59]. Recently, other signaling pathways besides the Smad proteins have also been shown to mediate TGF-β signaling in scleroderma fibroblasts, such as the p38 mitogen-activated protein kinase (MAPK) [40] and phosphatidylinositol 3-kinase (PI3K) [3] pathways. Signaling pathways involved in the

induction of scleroderma by bleomycin need future study.

Type 2 cytokines

Recent hypotheses have indicated that an imbalance exists between the type 1 and type 2 cytokine response in the pathogenesis of scleroderma. The contribution of IL-4 to scleroderma leads to the classification of this disorder as type 2 conditions. Additionally, a report shows that most CD4+ T cell clones generated from scleroderma skin biopsies exhibited type 2 cytokine profiles [69]. Serum in the majority of SSc patients showed elevated levels of CD30 [69], which is expressed on activated type 2 cells. IL-4 is produced by activated memory T cells and mast cells, both of which have a significant role in the pathogenesis of scleroderma. IL-4 is known to promote fibroblast proliferation, gene expression and synthesis of ECM proteins such as collagen and tenascin [65, 92]. IL-4 has recently been shown to upregulate the tissue inhibitor of metalloproteinase-2 (TIMP-2) in dermal fibroblasts via p38 MAPK pathway [39]. IL-4 upregulates TGF-β production in eosinophils [20] and T cells [103]. Increased IL-4 production is detected in the sera or by activated peripheral blood mononuclear cells of patients with SSc [78]. Scleroderma fibroblasts express more IL-4 receptor α and produce more collagen after IL-4 stimulation [105]. In the bleomycin model, IL-4 levels in the serum [125] as well as in the lesional skin [67] were significantly elevated following bleomycin treatment.

IL-13 is a pleiotropic cytokine, elaborated in significant quantities by appropriately stimulated type 2 cells. IL-13 has the ability to suppress proinflammatory cytokine production in monocytes/macrophages and is known to enhance the growth and differentiation of B cells and to promote immunoglobulin synthesis. In addition, in vitro studies demonstrated that IL-13 is a potent stimulator of fibroblast proliferation and collagen production [16, 46, 85]. IL-13 has been implicated in the pathogenesis of fibrotic conditions including SSc [32]. The profibrotic effect of IL-13 is postulated to involve irreversible fibroblast activation, triggered either directly [17] or indirectly through TGF-β [61, 85]. IL-13 transgenic mice show increased lung fibrosis, as well as increased levels of TGF-β1 [5]. In the bleomycin model, IL-13 mRNA levels in the lesional skin increased after bleomycin treatment [67]. Immunohistochemical localization showed enhanced expression of IL-13 on the infiltrating mononuclear cells in the lesional skin, parallel to the induction of dermal sclerosis, and IL-13 protein levels were also significantly greater. IL-13 receptor (IL-13R)-α2 expression in the lesional skin was augmented mainly in the infiltrating mononuclear cells and macrophages after bleomycin exposure. IL-13R-α2 mRNA level in the whole skin was upregulated, whereas IL-13R-α1 mRNA was not significantly enhanced. IL-13 may promote the progression of cutaneous fibrosis/sclerosis in the development of bleomycin-induced scleroderma.

Tumor necrosis factor-α

Expression of TNF is detectable during the very early stages of scleroderma [36]. The serologic level of TNF increases with the clinical severity and biologic activity of the disease [1], or in association with pulmonary fibrosis [33]. In vitro, bleomycin activates human alveolar macrophages to produce tumor necrosis factor-α (TNF-α). In vivo studies show increased levels of TNF-α protein and mRNA in lungs following bleomycin treatment in mice [88, 89]. The development of lung fibrosis was prevented by the administration of antibodies against TNF-α [89]. TNF-α exerts its biological effects through two TNF-α receptors; TNFR1 (55 kDa) and TNFR2 (75 kDa). TNFRp55-deficient mice developed severe sclerotic changes of the dermis following bleomycin exposure at extremely earlier time points, as compared with wild type mice [75]. Induction of matrix metalloproteinase-1 (MMP-1) expression was significantly inhibited in the bleomycin-treated skin of TNFRp55-null mice. The authors suggest that signaling mediated by TNFRp55 plays an essential role in MMP-1 expression and a key role in the collagen degradation process in the bleomycin model.

Role of chemokines in bleomycin-induced scleroderma

CCL2 is a chemoattractant for monocytes and T cells, belonging to a C–C chemokine superfamily of small proteins that are important in recruiting and activating leukocytes during inflammation [62]. Previous studies have shown that numerous types of cells including fibroblasts, endothelial cells, epithelial cells, mononuclear cells and smooth muscle cells are capable of expressing CCL2 in the presence of serum or specific stimuli [62]. Recent studies have demonstrated that CCL2 gene expression is upregulated in human fibrotic condition, as well as in animal models of fibrosis. In vitro studies show that CCL2 upregulates type I collagen mRNA expression in rat fibroblasts, which is indirectly mediated by endogenous upregulation of TGF-β gene expression [25]. CCL2 enhances expression of MMP-1, MMP-2 as well as TIMP-1 in cultured skin fibroblasts [129]. Current studies have demonstrated increased expression of CCL2 in patients with SSc [14, 23, 34, 132, 133]. Hasegawa et al. [34] demonstrated that serum levels and spontaneous production levels by peripheral blood mononuclear cells of CCL2 were elevated in patients with SSc compared with normal controls. Elevated serum CCL2 levels were correlated with pulmonary fibrosis. Immunohistochemical analysis also showed increased expression of CCL2 in scleroderma skin [14, 34, 133]. Scleroderma fibroblasts express increased levels

of CCL2 mRNA and protein [23, 132]. Distler et al. [14] reported that stimulation with PDGF resulted in a significant increase in CCL2 mRNA and protein. Furthermore, the autoinduction of CCL2 was observed in scleroderma fibroblasts, but not in normal fibroblasts [132]. These in vivo and in vitro results suggest an important involvement of CCL2 in the pathogenesis of scleroderma. CCL2 acts indirectly via IL-1α [129]. IL-1α as well as IL-1 receptor levels, in turn, were shown to be significantly increased in scleroderma [47]. In addition, IL-1α as well as TNF-α, are potent inducers of CCL2. Thus, in addition to a direct autocrine stimulatory loop, a mutual induction between IL-1α and CCL2 might lead to a self-perpetuating activation of scleroderma fibroblasts. CCL2 levels may also be increased by IL-13, because IL-13 is a potent stimulator of CCL2 [146].

As described before, increased numbers of mast cells are noted in scleroderma skin. CCL2 also recruits mast cells, in addition to monocytes [113]. Human mast cells are shown to be a rich source of chemokines, including CCL2, CCL3/macrophage inflammatory protein-1α (MIP-1α), CCL4/MIP-1β and CCL5/RANTES [104], as well as a number of cytokines/growth factors and mediators capable of activating fibroblasts or endothelial cells. Expression of stem cell factor (SCF), a mast cell growth factor, is upregulated in scleroderma fibroblasts [121]. SCF enhances CCL2 expression in human mast cells [134]. Because CCL2 enhances type I collagen mRNA expression in skin fibroblasts, the mutual interaction between mast cells and fibroblasts via SCF/CCL2 may play an important role in fibrosis.

In the bleomycin model, expression of CCL2 in the infiltrating mononuclear cells was enhanced following bleomycin treatment, and expression of CCL2 in fibroblasts was detected at later stages in the sclerotic skin [137]. Expression of CCR-2, a major receptor for CCL2, was also upregulated in the lesional skin at both protein and mRNA levels following bleomycin treatment. Administration of anti-CCL2 neutralizing antibody together with local bleomycin treatment reduced dermal sclerosis, along with collagen content in the skin as well as mRNA expression of type I collagen. These data suggest that CCL2 and CCR-2 signaling plays an important role in the pathogenesis of bleomycin-induced scleroderma. CCL2 may contribute to the induction of dermal sclerosis directly, via its upregulation of mRNA expression of ECM on fibroblasts, as well as indirectly through the mediation of a number of cytokines released from immunocytes recruited into the lesional skin.

Extracellular matrix

Excessive deposition of connective tissue is the result of an imbalance between synthesis and degradation. Maintenance of the normal balance of tissue turnover requires tight control of the activation of latent proenzymes and inhibition of proteolytic activity by TIMPs. TIMPs are specific inhibitors of MMPs activity, and suggested to be important for fibrogenesis. TIMP-1 expression in fibroblasts is regulated by several cytokines, among which TGF-β is the most important inducer. TIMP-1 has a growth stimulatory activity for skin fibroblasts [50]. Increased deposition of ECM proteins together with a decrease of collagenase activity in scleroderma skin may be related to elevated levels of serum TIMP-1 in patients with SSc [49]. Additionally, scleroderma fibroblasts produce increased amounts of TIMP-1 compared with normal fibroblasts [57, 112]. In the bleomycin model, TIMP-1 mRNA levels and concentrations were selectively upregulated in the lesional skin, whereas neither TIMP-1 nor TIMP-3 significantly changed (Yamamoto et al., manuscript in preparation).

Plasminogen activation is regulated by the balance between PAs and plasminogen activator inhibitors (PAIs). Recent findings suggest that PAI-1 is upregulated in a variety of fibrotic diseases, including scleroderma [15]. PAI-1 is strongly induced by TGF-β [48] and its promoter contains Smad binding elements. In the bleomycin model, PAI-1 expression at mRNA and protein levels, as well as protein production was upregulated in the lesional skin [67]. On the other hand, dermal sclerosis was similarly induced even in PAI-1-deficient mice [67]. Although PAI-1 null mice are protected from lung fibrosis [19], these results suggest that PAI-1 plays an important role, but is not the prerequisite factor, in the development of bleomycin-induced scleroderma.

Oxidant stress

Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions have multifarious effects that include oxidative damage to DNA. ROS can cause several abnormalities such as endothelial cell damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines including PDGF and TGF-β; thus excessive oxidative stress has been implicated in the pathogenesis of scleroderma [97]. Indeed, scleroderma fibroblasts produce ROS constitutively [98]. Other effects of oxygen radicals include the stimulation of skin fibroblast proliferation at low concentrations [76] and the production of increased amounts of collagen [21], suggesting that low oxygen tension may contribute to the increased fibrogenic properties of scleroderma fibroblasts. Furthermore, a study has demonstrated that several of the autoantigens targeted by scleroderma autoantibodies are fragmented in the presence of ROS and specific metals such as iron or copper [8]. The authors suggest that tissue ischemia generates such ROS, which induces the fragmentation of specific autoantigens. On the other hand, oxidative stress transiently induces CCL2 mRNA

and protein expression in cultured skin fibroblasts [24], suggesting that ROS may play a regulatory role in inflammation by modulating monocyte chemotactic activity.

Role of apoptosis

Involvement of the apoptotic process in the pathogenesis of SSc has been investigated [44, 99]. Endothelial cell apoptosis in particular is suggested to occur early in the pathogenesis of scleroderma. Endothelial cell apoptosis was first noted in the UCD 200/206 chickens that develop hereditary systemic connective tissue disease resembling human SSc [106]. This phenomenon is observed before perivascular mononuclear cell infiltration occurred. Also, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was shown to be positive on the endothelial cells in human scleroderma skin [99]. Recent studies have shown that apoptosis of endothelial cells induces resistance to apoptosis in fibroblasts largely through PI3K-dependent mechanisms [60]. Furthermore, fibroblasts exposed to medium conditioned by apoptotic endothelial cells presented myofibroblast changes [60]. By contrast, apoptosis of fibroblasts in SSc fibrotic skin lesions was not observed [24], and cultured scleroderma fibroblasts were resistant to Fas-induced apoptosis [44, 99]. Although the effect of TGF-β on apoptosis differs according to cell type, stage of maturation and other factors, TGF-β1 may play a role in inducing apoptosis-resistant fibroblast populations in SSc [99]. In scleroderma fibroblasts, Bcl-2 level was significantly higher, whereas the Bax level decreased significantly [99]. On the contrary, not only in Tsk1 but also Tsk2 mice, alterations in endothelial apoptosis induction were not involved in the development of the disease [107]. In the bleomycin model, TUNEL-positivity was prominently detected on keratinocytes and infiltrating mononuclear cells, but not endothelial cells and fibroblasts [140]. DNA fragmentation revealed laddering of the whole skin following bleomycin treatment. Increased expression of Fas was detected in infiltrating mononuclear cells at early phases, and FasL expression increased in mononuclear cells as well as fibroblasts in the sclerotic skin. Expression of FasL mRNA was upregulated by bleomycin treatment, whereas Fas mRNA was continuously detected. mRNA expression as well as activity of caspase-3 was also enhanced after bleomycin treatment. Administration of neutralizing anti-FasL antibody together with local bleomycin treatment reduced the development of dermal sclerosis, in association with the reduction of TUNEL-positive mononuclear cells and with the blockade of apoptosis. Caspase-3 activity was also significantly reduced after anti-FasL treatment. Excessive apoptosis, which is mediated by the Fas/FasL pathway and caspase-3 activation, is involved in the pathogenesis of bleomycin-induced scleroderma. Injection of anti-FasL antibody ameliorated the development of bleomycin-induced pulmonary fibrosis [58]. Abnormal regulation of apoptosis may contribute to the pathogenesis of scleroderma.

Therapeutic approach

Recent studies have demonstrated that blockade of TGF-β either by antibodies against TGF-β or by soluble TGF-β receptor, inhibits the development of tissue fibrosis in the experimental animal models [70, 118, 125]. In bleomycin-induced scleroderma, systemic administration of anti-TGF-β antibody, which cross-reacts with TGF-β1 and -β2, in combination with local bleomycin treatment, suppressed the development of scleroderma [125]. This effect was accompanied by the reduction of mast cell and eosinophil numbers. However, on account of the brief half-life of the antibody, repeated injections were required. Very recently, topical application of a peptide inhibitor of TGF-β has been shown to ameliorate skin fibrosis in bleomycin-induced scleroderma [100]. Future studies may address gene therapy targeting TGF-β signaling.

Interferons (IFNs), in particular IFN-γ, cause potent inhibition of collagen production, correlating with a reduction in corresponding steady-state mRNA levels in cultured skin fibroblasts [18, 45, 96]. IFN-γ decreased TGF-β-induced α-SMA expression in palatal fibroblasts, as well as changes in morphology [141]. Moreover, IFN-γ inhibits the TGF-β-induced phosphorylation of Smad3 and the accumulation of Smad3 in the nucleus, whereas induces the expression of Smad7, which prevents the interaction of Smad3 with the TGF-β receptor [114]. IFN-γ has been evaluated in clinical trials for use in the treatment of SSc, and long-term treatment with IFN-γ achieved moderate skin softening [38]. IFN-γ is a powerful type 1 inducer of cellular immunity, which may indirectly contribute to the improvement of the imbalance in the type 2 shift. In the bleomycin model, systemic administration of IFN-γ together with bleomycin reduced dermal sclerosis, even after the onset of scleroderma [130]. On the other hand, IFN-α did not suppress the dermal sclerosis induced by bleomycin.

Overproduction of ROS is commonly found in scleroderma patients with an active disease state [97]. Bleomycin is known to generate ROS, such as superoxide and hydroxyl radicals [81]. Therefore, a reduction of free radical formation may contribute to the decrease of collagen content by inhibition of proline hydroxylation, which leads to the improvement of scleroderma. We observed the inhibitory effect of lecithinized superoxide dismutase (SOD), which shows high tissue accumulation and a long half-life in the blood, on bleomycin-induced scleroderma, suggesting

that anti-oxidant therapy may lead to an anti-fibrotic effect [126]. However, post-onset administration of SOD could not attenuate the dermal sclerosis [126].

Halofuginone has an inhibitory effect on collagen synthesis, and shows anti-fibrotic effects in a few animal models of scleroderma. Halofuginone attenuates collagen synthesis, as well as collagen gene expression in avian and murine skin fibroblasts [26]. Halofuginone specifically inhibits α1(I) collagen gene expression without affecting the synthesis of other types of collagen such as type II and III [10, 64]. Halofuginone inhibited TGF-β-induced upregulation of collagen protein and α2(I) promoter activity, as well as phosphorylation and subsequent activation of Smad3 after TGF-β stimulation [71]. Dermal application of halofuginone on Tsk1/+ mice for 60 days reduced dermal fibrosis as well as collagen α1(I) gene expression [91]. Intraperitoneally administered halofuginone also prevented the thickening of the dermis and eliminated the increase of skin collagen in both Tsk1/+ and cGvHD models [10]. By contrast, bleomycin-induced scleroderma was not attenuated by treatment with halofuginone [136]. Halofuginone may not ameliorate dermal sclerosis along with strong inflammation caused by repeated application of bleomycin.

Hepatocyte growth factor (HGF) was originally identified and cloned as a potent mitogen for hepatocytes. Recent findings demonstrate that HGF prevented the progression of liver cirrhosis, renal fibrosis and pulmonary fibrosis. The promoter region of the HGF gene contains a TGF-β inhibitory element. HGF induces proteases, which degradate ECM proteins, such as MMPs, membrane type1-MMP (MT1-MMP) and urokinase-type plasminogen activator (uPA), and also decreases TIMPs [66]. Gene transfer of HGF not only prevented the ongoing dermal sclerosis induced by simultaneous local injections of bleomycin, but also ameliorated the previously induced dermal sclerosis [120]. This effect was mediated by suppressing TGF-β levels.

Perspective

Complex networks involve cell–cell and cell–matrix interactions via mediators in the induction of cutaneous sclerosis. Activated fibroblasts are a part of the immune system, and modulate immune cell behavior by conditioning the local cellular and cytokine microenvironment. We described here the characteristics of the experimental murine model of scleroderma induced by bleomycin. This model reproduces histological dermal sclerosis with cellular infiltration, vascular damage, lung fibrosis and the production of autoantibodies. The induction of dermal sclerosis is considered to be, in part, mediated by inflammatory and fibrogenic cytokines, as well as by the direct effect of bleomycin on ECM synthesis in fibroblasts. Additional mechanisms such as apoptosis and production of ROS are also speculated to be involved in the induction of scleroderma. It must be mentioned that the animal model is a simplification of the more complex human scleroderma. Nonetheless, the pathogenic mechanism discovered in the animal model may provide novel information, and assist in helping us to understand better, the mechanisms underlying human scleroderma. Animal models of scleroderma may also serve as promising tools for the development of new therapies specifically targeting individual cytokines, cytokine antagonists (i.e., antibodies, soluble receptors), cytokine mutants or of drugs that specifically interfere with the signal transduction pathways involved in the fibrotic process.

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